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BACTERIOLOGICAL STUDIES ON ALFALFA SILAGE

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INTRODUCTION

The practicability of making silage from alfalfa has been under investigation at this Station for several years. During the spring of 1914 the Dairy Department erected seven small stave silos, estimated to hold 10 tons each, for experimental purposes. The silos were filled with the following combinations:

Alfalfa alone.
Alfalfa 10 parts and corn chop 1 part.
Alfalfa 20 parts and blackstrap molasses 1 part.
Alfalfa 10 parts and alfalfa-molasses feed 1 part.
Alfalfa 4 parts and straw 1 part.
Alfalfa 2 parts and green rye 1 part.
Rye alone.

In the spring of 1915 the silos were again filled, but with the following combinations:

Alfalfa alone.
Alfalfa 20 parts and blackstrap molasses 1 part.
Alfalfa 10 parts and blackstrap molasses 1 part.
Alfalfa 10 parts and corn chop 1 part.
Alfalfa 6 parts and sweet-sorghum stover 1 part.
Alfalfa 2 parts and green rye 1 part.
Rye alone.

The exact ratio of the alfalfa and supplements in some cases varied slightly from the general plan outlined. Detailed information relative to this ratio and to filling the silos may be found in the report of Reed and Fitch (*15*).[1] Chemical analysis of the silage was made each year by the Department of Chemistry, while the microbial content was determined by the Department of Bacteriology.

Reed and Fitch (*15, p. 3*) concluded from these investigations that—

(1) Alfalfa will make a fairly good quality of silage, and it will be readily eaten by cattle if fed within a few months after being siloed.

[1] Reference is made by number (italic) to "Literature cited," p. 591-592.

(2) The addition of carbohydrate material, such as corn meal, blackstrap molasses, sweet-sorghum stover, and green rye, when put into the silo resulted in preserving it for a longer time than when alfalfa was siloed alone.
(3) Of the supplements used in these experiments blackstrap molasses proved to be the best, corn chop was next in order, followed by sweet sorghum stover and green rye.
(4) The mixture of alfalfa and blackstrap molasses was the most practical one used.
(5) There is as much acid produced in alfalfa silage as in kafir or cane silage.

In a preliminary report pertaining to this investigation, Swanson and Tague (*17, p. 292*) state that—

(1) Most of the acids present in alfalfa silage are produced in the first two weeks.
(2) The addition of supplements insures a more rapid and plentiful production of acids.
(3) Sugar present in the material used in making silage disappears very rapidly. Completely matured silage contains no sugar.
(4) Molasses was the most effective supplement.
(5) Alfalfa as it is put into the silo contains only a small amount of nitrogen in amino form.
(6) Alfalfa silage contains a large amount of nitrogen in the amino form. In good silage about one-third of the nitrogen is in this form, and in bad alfalfa silage amount is sometimes one-half of the total nitrogen.
(7) Most of the change of nitrogen into amino form takes place in the first 10 days.

BACTERIOLOGICAL INVESTIGATIONS IN 1914

METHODS OF PROCEDURE

It was planned to collect samples for analyses at the time of filling the silo, each day after filling for seven days, every other day for the next week, every four days for the following two weeks, then once a week, and finally once a month until the silo was opened. While circumstances came up which interfered with this schedule at various times, this general plan of procedure was followed. The silage for analysis was obtained from the silos from a small hole about 2 inches in diameter bored into the side of the silo about 2 or 3 feet from the level of the ground. The samples of silage were collected in sterile containers by means of a large extension auger. After the required amount was obtained, the holes were tightly stoppered with paraffined corks. The general practice was to collect the samples, as aseptically as possible, from new holes each time. They were removed to the laboratory and examined immediately after collecting them.

BACTERIOLOGICAL TECHNIC

Twenty gm. of the silage were placed in 200 cc. of a sterile physiological-salt solution and thoroughly shaken. The necessary dilutions were made according to standard methods.

Plain agar was used for determining the total number of microorganisms.

Glucose-acetic-acid agar was used to determine the total number of the Bulgarian types of organisms present. The medium was 1 per cent

glucose agar, to which was added 1 cc. of a 1 per cent sterile acetic-acid solution. The acid solution was added directly to the plates and the glucose agar was mixed with it when the plates were poured. Lactose agar was used in place of glucose agar in the preliminary work, but from subsequent work it was observed that the glucose medium favored this type of organisms more than the lactose agar. The small amount of acid added was sufficient to check practically all types of organisms except the Bulgarian group and the yeasts. After a little experience there was no difficulty in distinguishing between these two types, on account of their characteristic colonies.

Litmus-lactose agar was used to determine total numbers, as well as the acid and neutral types, of microorganisms.

Bile lactose fermentation tubes were employed in determining colon organisms. The Dunham fermentation tubes were inoculated with different dilutions of the silage extract. The tube with the highest dilution showing gas production was used as an estimate of the total number of organisms of the colon group present. To substantiate further this presumptive test, different dilutions were plated out from time to time, and the organisms of the colon type isolated and identified.

Glucose fermentation tubes were used in determining the total number of yeasts present by noting gas production in the different dilution tubes. To be certain that the gas was due to yeast fermentation, stained preparations were made from each dilution and examined for the presence of yeast cells. If gas was present and no yeast could be demonstrated, it was taken for granted that the gas formation was not due to yeasts, but to other causes. The yeast count from the acid agar was used to check this method, and they compared very favorably. The general rule, however, was that higher numbers were obtained from the fermentation tubes than from the plates.

All media used were made from Liebig's beef extract, and a reaction of +1.0 to phenolphthalein required. The period of incubation, unless otherwise stated, was always four days at 37.5° C. The long period of incubation was used to favor the complete development of the Bulgarian type. The enumeration of all plates was done by the aid of a hand lens.

The principal and predominating types of microorganisms as they appeared on the different media from time to time were isolated. A morphological, cultural, and biochemical study was made from the organisms thus isolated.

Stained preparations were made directly from the silage infusion in order to check the results of the cultural analysis. It seemed unnecessary to employ the use of synthetic media after comparing the microscopic appearance of the silage infusion with the results obtained from the culture media, as the media gave a very good estimate of the true microbial content of the silage.

EXPERIMENTAL DATA

The silos were filled, beginning on May 23, 1914, with the first cutting of alfalfa, and were opened in January, 1915. The condition of the silage, when the silos were opened, is well described by Reed and Fitch (*15, p. 10–11*):

> In most cases the silage had settled five or six feet from the top of the silo and had drawn away slightly from the wall at the surface. The exceedingly dry, hot weather of 1914 perhaps caused more drying than would have occured during a normal year. The upper two feet of silage was spoiled in all the silos. The silage made from alfalfa alone was very dark in color and it had a disagreeable odor, which is characteristic of alfalfa when preserved in the silo. The mixtures of alfalfa and corn chop, alfalfa and molasses, alfalfa and molasses feed, all showed a dark-green color. All of the mixtures had a strong odor. The moisture content of all mixtures except the alfalfa and rye was very low. * * * The mixtures of alfalfa and straw, and alfalfa and molasses feed did not make a good quality of silage. On the whole, the results obtained in this trial were not satisfactory.

The palatability test as determined by the Dairy Department demonstrated that cattle prefer the mixtures in the following order: Alfalfa and molasses, alfalfa and corn chop, alfalfa alone, alfalfa and rye, alfalfa and molasses feed, alfalfa and straw, and rye alone.

The acidity of the silage as determined by the Chemistry Department indicated that all the material underwent a normal acid fermentation. The acidity for the silage, when the silos were opened was as follows: Alfalfa alone 1.63 per cent, alfalfa and corn chop 1.84 per cent, alfalfa and molasses 2.28 per cent, alfalfa and molasses feed 2.03 per cent, alfalfa and straw 1.37 per cent, alfalfa and rye 1.24 per cent, and rye alone 2.79 per cent.

The results obtained from the bacteriological analyses are given in Table I.

In general, the bacteriological data were unsatisfactory. Little evidence was gathered to account for the differences noted in the quality of the various kinds of silage. The microbial flora of alfalfa silage is very similar to that of silage made from the common forage crops, the four principal types of organisms being (1) acid producers, (2) yeasts, (3) organisms of the colon group, and (4) miscellaneous. As in normal silage, the acid producers, as a rule, predominated in alfalfa silage. Yeasts and the organisms of the colon group had a tendency to coincide in their course of development in the silage. It has been observed from the study of the fermentation processes in a good quality of silage that these two groups rapidly increase in numbers for the first few days, then gradually diminish. While the same tendency was indicated in the different kinds of alfalfa silage, it was more marked in some than in others.

TABLE I.—*Microbial content of various kinds of silage—Experiments of 1914*

ALFALFA ALONE

Date.	Days.	Number of microorganisms per gram upon—					Number of organisms of colon type per gram.	Number of yeasts per gram.
		Plain agar—Total number.	Litmus-lactose agar.			Acid agar—Acid type.		
			Total.	Acid producers.	Neutral type.			
May 23	0	3,000,000	520,000	380,000	140,000	0	10,000	10,000
May 25	2	43,000,000	48,000,000	46,000,000	2,000,000	285,000	1,000,000	10,000,000
May 26	3	25,000,000	.....	.....	.....	7,000	1,000	1,000,000
May 27	4	.....	10,000,000	9,000,000	1,000,000	0	10,000	1,000,000
May 28	5	.....	.....	.....	.....	70,000	10,000	10,000
May 29	6	17,000,000	14,000,000	14,000,000	0	42,000	1,000	10,000
May 30	7	8,500,000	5,000,000	5,000,000	0	300,000	100	1,000
June 1	9	16,000,000	5,500,000	4,500,000	1,000,000	4,000	10	1,000
June 2	10	10,000,000	8,000,000	8,000,000	0	120,000	100	1,000
June 3	11	11,000,000	.....	.....	.....	400,000	100	1,000
June 4	12	7,000,000	5,500,000	5,500,000	500,000	24,000	0	100
June 5	13	8,000,000	10,000,000	8,000,000	2,000,000	500,000	1,000	1,000
June 6	14	4,000,000	3,000,000	2,500,000	500,000	10,000	10	10
June 8	16	17,000,000	10,000,000	5,000,000	5,000,000	4,000	10	10
June 9	17	500,000	600,000	300,000	300,000	.....	10	10
June 10	18	10,000,000	13,000,000	11,000,000	1,000,000	.....	100	10
June 11	19	2,000,000	2,000,000	1,800,000	200,000	20,000	0	100
June 15	23	10,000,000	800,000	400,000	400,000	18,000	0	10
June 17	25	.....	.....	.....	.....	23,000	100	100
June 19	27	11,000,000	10,000,000	800,000	9,400,000	12,000	10	100
June 22	30	30,000,000	9,500,000	8,000,000	1,500,000	500,000	10,000	10,000
June 26	34	30,000,000	500,000,000	200,000,000	300,000,000	150,000	0	0
July 3	41	4,500,000	3,000,000	2,500,000	500,000	50,000	1,000	1,000
July 10	48	1,500,000	1,000,000	1,200,000	400,000	2,000	0	0
July 17	55	300,000	100,000	0	100,000	4,000	10	10
July 28	66	10,000,000	30,000,000	22,000,000	8,000,000	2,000	.....	10
September 18	118	.....	.....	.....	.....	450,000	10	10
October 2	132	500,000,000	500,000,000	250,000,000	250,000,000	550,000	10,000	10,000
October 30	160	50,000,000	30,000,000	5,000,000	25,000,000	2,000,000	10,000	10,000

TABLE I.—*Microbial content of various kinds of silage—Experiments of 1914*—Continued

ALFALFA AND CORN CHOP

Date.	Days.	Number of microorganisms per gram upon—					Number of organisms of colon type per gram.	Number of yeasts per gram.
		Plain agar—Total number.	Litmus-lactose agar.			Acid agar—Acid type.		
			Total.	Acid producers.	Neutral type.			
May 25	0	63,000,000	38,000,000	0	73,000,000	700	10,000	10,000
May 26	1	87,000,000	38,000,000	0	33,000,000	0	10,000	10,000
May 27	2	.....	7,000,000	2,000,000	5,000,000	5,000	1,000	1,000
May 28	3	15,000,000	.....	.....	.....	120,000	10,000	10,000
May 29	4	10,000,000	17,000,000	15,000,000	2,000,000	67,000	1,000,000	1,000,000
May 30	5	29,000,000	27,000,000	5,000,000	22,000,000	300,000	100,	1,000
June 1	7	.....	9,000,000	8,500,000	500,000	50,000	100	100
June 2	8	30,000,000	20,000,000	20,000,000	0	14,000	100	10,000
June 3	9	5,000,000	.....	.....	.....	100,000	100,	1,000
June 4	10	6,500,000	20,000,000	10,000,000	10,000,000	18,000	100	100
June 5	11	8,000,000	6,000,000	2,000,000	4,000,000	69,000	10	100
June 6	12	80,000,000	50,000,000	35,000,000	15,000,000	1,000	10	10
June 8	14	13,000,000	18,000,000	7,500,000	10,500,000	59,000	100	100
June 9	15	600,000	5,000,000	3,000,000	2,000,000	3,000	100	10
June 10	16	2,000,000	2,500,000	2,000,000	500,000	120,000	0	0
June 11	17	10,000,000	11,000,000	9,000,000	2,000,000	15,000	1,000	10
June 15	21	400,000	500,000	100,000	400,000	200,000	0	100
June 17	23	.....	8,500,000	7,000,000	1,500,000	1,600,000	10,000	10,000
June 19	25	7,000,000	6,000,000	4,500,000	1,500,000	100,000	100	100
June 21	28	10,000,000	7,000,000	5,500,000	1,500,000	10,000	10	10
June 26	32	15,000,000	9,000,000	3,000,000	6,000,000	20,000	0	0
July 3	39	10,000,000	9,500,000	7,500,000	3,000,000	200	0	0
July 10	46	400,000	500,000	200,000	300,000	200	0	0
July 17	53	1,000,000	1,300,000	300,000	1,000,000	0	100	100
July 23	64	600,000	190,000	0	190,000	100	0	10
September 18	116	3,000,000	900,000	100,000	800,000	200,000	1,000	1,000
October 2	130	170,000,000	230,000,000	30,000,000	200,000,000	60,000	10	100
October 30	158	200,000,000	200,000,000	50,000,000	150,000,000	120,000	100	1,000

ALFALFA AND MOLASSES

May 26	0	.....	.....	.....	.....	0	1,000	1,000
May 27	1	.....	90,000,000	85,000,000	5,000,000	0	10,000	1,000,000
May 28	2	8,000,000	.....	.....	.....	300,000	10,000	10,000
May 29	3	31,000,000	18,000,000	17,000,000	1,000,000	12,000	1,000	1,000
May 30	4	30,000,000	33,000,000	32,000,000	1,000,000	2,500,000	1,000	10,000
June 1	6	.....	2,000,000	1,500,000	500,000	350,000	10	100
June 2	7	23,000,000	28,000,000	28,000,000	0	10,000	1,000	10,000

June 3	8	27,000,000	....	....	....	400,000	10,000	10,000
June 4	9	70,000,000	40,000,000	20,000,000	20,000,000	200,000	0	100
June 5	10	8,000,000	7,000,000	5,000,000	2,000,000	75,000	10	100
June 6	11	14,000,000	4,000,000	4,000,000	0	50,000	10	10
June 8	13	12,000,000	3,500,000	3,500,000	0	70,000	10	10
June 9	14	4,000,000	600,000	500,000	100,000	110,000	10	100
June 10	15	12,000,000	13,000,000	10,000,000	3,000,000	150,000	1,000	100
June 11	16	....	1,000,000	1,000,000	0	75,000	0	0
June 15	20	5,000,000	7,500,000	1,000,000	6,500,000	300,000	10	100
June 17	22	....	....	....	....	800,000	1,000,000	1,000,000
June 19	24	2,000,000	5,500,000	5,500,000	0	300,000	0	100
June 22	27	900,000	200,000	200,000	0	600,000	0	0
June 26	31	400,000	300,000	0	300,000	31,000	0	0
July 3	38	50,000	20,000	10,000	10,000	1,100	0	0
July 10	45	45,000	50,000	40,000	10,000	200	0	0
July 17	52	1,300,000	400,000	0	400,000	0	0	0
July 18	63	100,000	....	....	....	0	0	0
September 18	115	2,000,000,000	375,000,000	0	375,000,000	150,000	100	10
September 2	129	250,000	20,000	0	20,000	40,000	0	0
October 30	157	200,000,000	175,000,000	10,000,000	165,000,000	250,000	10,000	10,000

ALFALFA AND ALFALFA-MOLASSES FEED

May 27	0	....	4,000,000	1,000,000	3,000,000	0	100	1,000
May 28	1	67,000,000	....	....	....	11,000	1,000,000	1,000,000
May 29	2	160,000,000	1,000,000,000	700,000,000	300,000,000	60,000	10,000	10,000
May 30	3	26,000,000	48,000,000	30,000,000	18,000,000	2,000,000	100	10
June 1	5	....	40,000,000	15,000,000	25,000,000	3,000,000	1,000	1,000
June 2	6	16,000,000	65,000,000	65,000,000	0	100,000	1,000	1,000
June 3	7	5,000,000	....	....	....	600,000	10	100
June 4	8	15,000,000	23,000,000	14,000,000	9,000,000	300,000	0	100
June 5	9	15,000,000	18,000,000	14,000,000	4,000,000	100,000	100	100
June 6	10	35,000,000	20,000,000	15,000,000	5,000,000	5,000	10	10
June 8	12	15,000,000	6,000,000	4,500,000	1,500,000	200,000	10	10
June 9	13	1,600,000	4,000,000	2,000,000	2,000,000	100,000	100	100
June 10	14	....	8,000,000	6,000,000	2,000,000	75,000	100	1,000
June 11	15	4,000,000	1,700,000	1,000,000	700,000	....	0	100
June 15	19	1,500,000	1,000,000	200,000	800,000	600,000	10	10
June 17	21	....	....	....	....	300,000	10,000	1,000,000
June 19	23	10,000,000	30,000,000	20,000,000	10,000,000	7,500,000	1,000	10,000
June 22	26	3,000,000	4,000,000	3,500,000	500,000	200,000	0	0
June 26	30	3,500,000	5,000,000	2,000,000	3,000,000	150,000	10	1,000
July 3	37	1,500,000	500,000	0	500,000	5,000	0	0
July 10	44	1,000,000	....	....	....	0	100	0
July 17	51	1,600,000	1,000,000	0	1,000,000	0	1,000	1,000
July 28	62	500,000	200,000	0	200,000	0	100	100
September 18	114	2,000,000	3,000,000	1,250,000	1,750,000	60,000	10	100
October 2	128	7,000,000	5,000,000	0	5,000,000	40,000	0	1,000
October 30	156	25,000,000	30,000,000	5,000,000	25,000,000	20,000	10	10

TABLE I.—*Microbial content of various kinds of silage—Experiments of 1914*—Continued

ALFALFA AND STRAW

Date.	Days.	Number of microorganisms per gram upon—						
		Plain agar—Total number.	Litmus-lactose agar.			Acid agar—Acid type.	Number of organisms of colon type per gram.	Number of yeasts per gram.
			Total.	Acid producers.	Neutral type.			
May 27	0	122,000,000				0	10,000	
May 28	1					30,000	1,000,000,000	1,000,000,000
May 29	2	75,000,000,000	70,000,000,000	60,000,000,000	10,000,000,000	1,000,000	1,000,000,000	10,000,000,000
May 30	3	20,000,000,000	50,000,000,000	40,000,000,000	10,000,000,000	6,000,000	10,000,000	1,000,000,00
June 1	5	1,000,000,000	1,500,000,000	1,000,000,000	500,000,000	700,000	1,000,000	10,000,000
June 2	6	800,000,000	15,000,000,000	14,000,000,000	1,000,000,000	100,000	10,000	1,000,000
June 3	7	4,000,000,000				800,000	10,000	1,000,000
June 4	8	470,000,000	600,000,000	600,000,000	0	2,200,000	10,000	1,000,000
June 5	9	300,000,000	580,000,000	580,000,000	0	5,200,000	10,000	10,000
June 6	10	2,000,000,000	2,000,000,000	1,250,000,000	150,000,000	500,000	1,000	10,000
June 8	12	230,000,000	300,000,000	250,000,000	50,000,000	175,000	1,000	10,000
June 9	13	450,000,000	500,000,000	480,000,000	20,000,000	40,000	10,000	10,000
June 10	14		70,000,000	70,000,000	0	240,000	1,000	1,000
June 11	15	370,000,000	70,000,000	60,000,000	10,000,000	15,000	10,000	10,000
June 15	19	80,000,000	90,000,000	90,000,000	0	400,000	100	10,000
June 17	21		1,500,000	1,200,000	300,000	200,000	10,000	1,000,000
June 19	23	200,000,000	50,000,000	150,000,000	200,000,000	3,100,000	1,000,000	1,000,000
June 22	26	10,000,000	40,000,000	40,000,000	0	1,000,000	100	100
June 26	30	16,000,000	18,000,000	10,000,000	8,000,000	100,000	100	100
July 3	37	30,000,000	40,000,000	20,000,000	20,000,000	5,000	1,000	1,000
July 10	44	2,500,000	1,000,000	500,000	500,000	0	100	100
July 17	51	1,500,000	500,000	0	0	500	1,000	1,000
July 28	62	2,000,000	2,000,000	1,000,000	1,000,000	0	0	100
September 18	114	3,000,000	2,000,000	400,000	1,600,000	8,000,000	1,000	1,000
October 2	128	7,000,000	900,000	0	900,000	8,000	10	10,000
October 30	156	25,000,000	20,000,000	5,000,000	15,000,000	10,000	10	1,000

ALFALFA AND RYE

May 28	0	15,000,000,000	10,000,000,000	34,000,000,000	24,000,000,000	13,000	1,000,000,000	10,000,000,000
May 29	1	500,000,000	400,000,000	300,000,000	100,000,000	19,000,000	10,000,000	10,000,000
May 30	2	1,200,000,000	2,700,000,0000	1,700,000,000	1,000,000,0000	20,000,000	10,000,000	10,000,000
June 1	4	20,000,000	45,000,000	25,000,000	20,000,000	1,000,000	10,000	1,000
June 2	5	85,000,000	50,000,000	45,000,000	5,000,000	3,000,000	10,000,000	10,000,000

June 3	6	75,000,000				8,500,000	1,000,000	10,000,000
June 4	7	250,000,000	150,000,000	60,000,000	90,000,000	7,500,000	1,000	10,000
June 5	8	50,000,000	20,000,000	2,000,000	18,000,000	1,200,000	100	100
June 6	9	750,000,000	8,000,000,000	400,000,000	7,600,000,000	12,000	10,000	10,000
June 8	11	230,000,000	80,000,000	40,000,000	40,000,000	1,200,000	10,000	10,000
June 9	12	22,000,000	30,000,000	0	30,000,000	1,800,000	10,000	10,000
June 10	13		30,000,000	0	30,000,000	6,000,000	100	1,000
June 11	14	1,000,000,000	1,000,000,000	500,000,000	500,000,000	6,000,000	10,000	10,000
June 15	18	36,000,000	30,000,000	0	30,000,000	11,000,000	0	0
June 17	20		6,000,000	0	6,000,000	14,000,000	0	100
June 19	22	3,000,000	350,000,000	50,000,000	300,000,000	7,500,000	10	100
June 22	25	20,000,000	20,000,000	0	20,000,000	200,000	10	100
June 26	29	10,000,000	10,000,000	0	10,000,000	600,000	0	0
July 3	36	3,500,000	2,600,000	200,000	2,400,000	16,000	100	100
July 10	43	3,000,000	2,500,000	0	2,500,000	0	0	0
July 17	50	5,000,000	5,000,000	2,000,000	3,000,000	3,000	10	1,000
July 28	61	3,000,000	6,000,000	0	6,000,000	0	0	10
September 18	113	17,000,000	8,000,000	0	8,000,000	50,000	10	100
October 2	127	37,000,000	12,000,000	0	12,000,000	30,000	0	100
October 30	155	10,000,000	5,000,000	3,000,000	2,000,000	50,000	0	0

RYE

May 28	0	60,000,000	33,000,000	18,000,000	15,000,000	3,500	10,000	10,000,000
May 29	1	250,000,000	200,000,000	190,000,000	10,000,000	9,500,000	1,000,000	10,000,000
May 30	2	700,000,000	3,000,000,000	2,000,000,000	1,000,000,000	100,000,000	1,000,000	1,000,000,000
June 1	4	300,000,000	200,000,000	100,000,000	100,000,000	12,000,000	1,000,000	10,000,000
June 2	5	50,000,000	58,000,000	58,000,000	0	4,000,000	10,000	10,000
June 3	6	85,000,000				40,000,000	10,000	10,000
June 4	7	60,000,000	29,000,000	16,000,000	13,000,000	6,500,000	10,000	10,000
June 5	8	35,000,000	6,500,000	4,500,000	2,000,000	3,000,000	10,000	10,000
June 6	9	55,000,000	25,000,000	12,000,000	13,000,000	1,500,000	10,000	10,000
June 8	11	20,000,000	2,500,000	1,500,000	1,000,000	1,500,000	10,000	1,000,000
June 9	12	15,000,000	1,000,000	900,000	100,000	400,000	1,000	1,000
June 10	13		24,000,000	23,500,000	500,000	600,000	10,000	10,000
June 11	14	35,000,000	10,000,000	10,000,000	0	2,000,000	10	1,000
June 15	18	5,000,000	1,800,000	1,000,000	800,000	2,000,000	1,000,000	10,000
June 17	20		20,000,000	10,000,000	10,000,000	8,500,000	1,000,000	1,000,000
June 19	22	500,000,000	450,000,000	430,000,000	20,000,000	40,000,000	1,000,000	1,000,000
June 22	25		60,000,000	40,000,000	20,000,000	5,000,000	1,000,000	10,000
June 26	29	3,500,000	7,000,000	6,000,000	1,000,000	6,000,000	10,000	10,000
July 3	36	8,000,000	9,000,000	8,500,000	500,000	40,000	1,000,000	1,000,000
July 10	43	1,500,000	400,000	300,000	100,000	25,000	10	100
July 17	50	6,000,000	1,300,000	400,000	900,000	25,000	10,000	10,000
July 28	61	1,000,000	35,000	27,000	8,000	10,000	10	10,000
September 18	113	2,000,000,000	80,000,000	30,000,000	50,000,000	40,000	100	
October 2	127	5,000,000	2,500,000	1,000,000	1,500,000	4,000,000		
October 30	155	45,000,000	20,000,000	15,000,000	5,000,000	10,000,000	1,000	1,000

It has likewise been demonstrated from the study of many classes of silage of high quality that the general course of development of the total microbial flora is slow to rapid, usually covering a period of a few days to two weeks, followed by a gradual decline. The data in Table I indicate a like tendency, but the characteristic development is not so pronounced as would be expected from a good grade of silage. This is partially explained, however, by the method used in sampling and also by the small capacity of the silos used, which did not favor optimum silage fermentation. The method of sampling, no doubt, accounts for a large portion of the variations noted, since the method did not always allow the collecting of proportionate amounts of leaves and stem, or where supplements were added of proportionate amounts of alfalfa and the supplement.

BACTERIOLOGICAL INVESTIGATIONS IN 1915

The seven silos were again filled in the spring of 1915 with alfalfa and the supplements previously mentioned. The general plan of study was the same as for 1914.

However, glucose-litmus broth was used to determine the total number of acid producers. It was prepared by using 1 per cent glucose broth to which a few drops of litmus solution had been added. Several tubes of this medium were inoculated with different dilutions of the silage infusion and incubated. The total acid producers were determined by noting the acid reaction in the highest dilution present.

This method was used in place of a litmus-agar medium because a more accurate estimate of the acid producers could be obtained. If on a litmus-agar plate the acid colonies are few and other types predominate, the colonies may fail to appear acid on account of the neutralization of the acid by the alkaline by-products from the other types. The organisms in the glucose-broth solution are not held in one place, as they are in the solid-agar media. This, together with the fact that the glucose broth acts as an enrichment medium for the acid group, gives better opportunity for the acid bacteria to increase more rapidly than the miscellaneous organisms. Likewise, the acid produced from the more rapid-growing acid bacteria is sufficient to check the slower development of the miscellaneous types. The dilution method is more tedious, but, provided the differences between the dilutions are small, the results obtained are more satisfactory and more accurate. Plain gelatin was used to determine the protein-digesting types. Not having a satisfactory place for the incubation of gelatin plates, sterile tubes of gelatin were inoculated with varying dilutions of silage, and incubated at 37.5° C. for five days. The number of liquefiers were determined by placing the gelatin tubes in the ice box, after their removal from the incubator. From previous experience it has been found that a tube of digested gelatin will not solidify on cooling. Hence, by noting the highest dilution [illegible] the number of liquefiers can be esti-

mated. By the use of small dilutions and more tubes, the results are probably more accurate than those obtained from the usual method of counting the liquefying colonies on gelatin plates.

EXPERIMENTAL DATA

The silos were opened on December 20, 1915, six and one-half months after being filled. The silage from all silos was of much better quality than that obtained the previous year. This was due, no doubt, to the greater care taken in packing and filling the silos (*15, p. 13–15*) and also to the supplements used. The strong offensive odor common to the silage produced in the first year's work was present only in the alfalfa and in the alfalfa-and-rye silage. The palatability test as determined by the Dairy Department demonstrated the feeding quality of the silage to be as follows: Alfalfa and molasses 20 to 1, alfalfa to molasses 10 to 1, alfalfa and corn chop, alfalfa and sweet-sorghum butts, alfalfa and rye, alfalfa alone, and rye alone.

The data obtained by the Chemistry Department, published by Swanson and Tague (*17*), failed to show the relative differences between the different kinds of silage. The alfalfa silage, like that in the previous year's work, exhibited the lowest acid content. The greatest, and practically the only characteristic difference observed from the chemical data was the total amount of acid produced. The acidity of alfalfa silage, calculated as lactic acid, on the last day examined, when 211 days old, was 1.72 per cent; alfalfa and molasses 20 to 1, 208 days old, 2.89 per cent; alfalfa and molasses 10 to 1 206 days old, 3.55 per cent; alfalfa and corn chop 10 to 1, 204 days old, 3.36 per cent; alfalfa and sweet-sorghum straw 6 to 1, 198 days old, 2.19 per cent; alfalfa and rye 2 to 1, 198 days old, 2.5 per cent; rye alone, 198 days old, 1.95 per cent.

The calculated percentage of amino nitrogen failed to exhibit any characteristic difference between the various types of silage.

The bacteriological results are tabulated in Table II.

TABLE II.—*Microbial content of various kinds of silage—Experiments of 1915*

ALFALFA

Date.	Days.	Number of microorganisms per gram of silage.					
		Total.	Total acid producers.	Bulgarian group.	Gelatin liquefiers.	Colon type.	Yeasts.
May 17.....	0	575,000	1,000,000	0	1,000	1,000	100
18.....	1	40,000,000	100,000	2,000	1,000	1,000,000	300
19.....	2	300,000,000	100,000	10,000	1,000	1,000,000	1,000
21.....	4	260,000,000	100,000,000	3,000,000	1,000	1,000,000	1,000
24.....	7	640,000,000	1,000,000,000	60,000,000	10,000	1,000,000	1,000,000
26.....	9	250,000,000	100,000,000	80,000,000	10,000	1,000	10,000
29.....	12	100,000,000	1,000,000,000	90,000,000	1,000	1,000	1,000
June 1.....	15	120,000,000	1,000,000,000	100,000,000	10,000	100	0
5.....	19	75,000,000	100,000,000	45,000,000	1,000	0	0
12.....	26	60,000,000	100,000,000	50,000,000	10,000	0	100
18.....	32	24,000,000	10,000,000	10,000,000	1,000,000	0	10,000
July 2.....	46	75,000,000	10,000,000	2,000,000	10,000	0	1,000
Aug. 6.....	81	24,000,000	100,000,000	2,000,000	10,000,000	0	10,000
28.....	103	60,000,000	100,000,000	8,000	10,000	0	100
Oct. 16.....	152	10,000	1,000	.....	1,000	0	100
Dec. 20.....	217	1,000,000	100,000	800	100	0	1,000

TABLE II.—*Microbial content of various kinds of silage—Experiments of 1915*—Contd.

ALFALFA AND MOLASSES 20 TO 1

Date.	Days.	Number of microorganisms per gram of silage.					
		Total.	Total acid producers.	Bulgarian group.	Gelatin liquefiers.	Colon type.	Yeasts.
May 19	0	4,000,000	.....	0	1,000	10,000	100,000
20	1	70,000,000	1,000,000,000	290,000	.....	1,000,000	1,000,000
21	2	200,000,000	10,000,000	3,000,000	100,000	10,000,000	10,000
24	5	900,000,000	1,000,000,000	25,000,000	1,000	1,000,000	10,000,000
26	7	400,000,000	1,000,000,000	300,000,000	.....	0	0
29	10	100,000,000	100,000,000	75,000,000	100	0	100
June 1	13	18,000,000	1,000,000,000	20,000,000	100,000	1,000	1,000
5	17	40,000,000	10,000,000	40,000,000	10,000	1,000	100
12	24	50,000,000	100,000,000	30,000,000	1,000	100	1,000
18	30	3,000,000	10,000,000	3,000,000	1,000	0	0
July 2	44	10,000,000	1,000,000	1,000,000	1,000	0	0
Aug. 6	97	400,000	10,000,000	3,000,000	10,000	0	0
28	101	520,000	1,000,000	1,000	100,000	0	0
Oct. 16	150	15,000	1,000	11,000	10,000	0	1,000
Dec. 20	215	50,000,000	100,000	5,000,000	10,000	0	0

ALFALFA AND MOLASSES 10 TO 1

May 22	0	1,000,000	10,000,000	15,000	10,000	100,000	1,000
May 24	2	1,000,000,000	1,000,000,000	50,000,000	1,000	100,000	10,000
May 25	3	900,000,000	100,000,000	100,000,000	10,000	1,000	100
May 26	4	500,000,000	1,000,000,000	300,000,000	10,000	0	0
May 29	7	100,000,000	10,000,000	120,000,000	100,000	0	0
June 1	10	14,000,000	1,000,000,000	30,000,000	10,000	0	0
June 5	14	11,000,000	10,000,000	3,000,000	100,000	0	0
June 12	21	50,000,000	10,000,000	2,000,000	10,000	0	100
June 18	27	200,000	100,000	400,000	100,000	0	1,000
July 2	41	500,000	100,000	30,000	10,000	0	1,000
August 6	76	1,000,000	100,000	700,000	10,000	0	0
August 28	98	3,000,000	1,000,000	9,000,000	10,000	0	0
October 16	147	50,000	0	100	10,000	0	0
December 20	212	300,000	100	20,000	1,000	0	0

ALFALFA AND CORN CHOP

May 24	0	2,000,000	100,000	150,000	100,000	100,000	10,000
May 25	1	1,000,000,000	1,000,000,000	20,000,000	10,000	1,000,000,000	1,000,000
May 26	2	2,000,000,000	100,000,000	200,000,000	10,000,000	10,000	10,000
May 29	5	530,000,000	1,000,000,000	400,000,000	1,000	1,000	1,000
June 1	8	250,000,000	10,000,000,000	100,000,000	10,000	0	100
June 5	12	120,000,000	10,000,000,000	120,000,000	10,000	0	100
June 12	19	140,000,000	100,000,000	20,000,000	10,000	100	0
June 18	25	16,000,000	10,000,000	40,000,000	1,000	0	100
July 2	39	250,000,000	10,000,000	14,000,000	10,000	0	0
August 6	74	1,000,000	1,000,000	3,000,000	10,000	1,000	1,000
August 28	96	10,000,000	100,000	90,000	100,000	0	0
October 16	145	800,000	1,000	80,000	.....	0	10,000
December 20	210	15,000,000	100,000	1,000,000	100,000	0	0

ALFALFA AND SWEET-SORGHUM STOVER

May 31	0	120,000,000	10,000,000	70,000	10,000	10,000	1,000,000
June 1	1	200,000,000	100,000,000	10,000,000	10,000	100,000	10,000,000
June 2	2	500,000,000	1,000,000,000	600,000,000	1,000,000	10,000,000	10,000,000
June 4	4	2,000,000,000	1,000,000,000	10,000	10,000,000	1,000,000	1,000,000
June 7	7	1,000,000,000	100,000,000	900,000,000	1,000	10,000	10,000
June 9	9	600,000,000	100,000,000	250,000,000	10,000	100,000	10,000
June 11	11	240,000,000	100,000,000	100,000,000	100,000	0	100
June 14	14	250,000,000	10,000,000	50,000,000	100,000	1,000	10,000
June 28	35	50,000,000	10,000,000	25,000,000	10,000,000	1,000	10,000
July 2	39	60,000,000	10,000,000	15,000,000	1,000,000	10,000	10,000
August 6	67	15,000,000	1,000,000	700,000	100,000	0	1,000
August 28	89	120,000,000	1,000,000	30,000,000	10,000	10,000	10,000
October 16	138	5,000,000	100,000	200,000	100	10,000	1,000
December 20	203	20,000,000	100,000,000	100,000	500,000	100,000	100,000

TABLE II.—*Microbial content of various kinds of silage—Experiments of 1915*—Contd.

ALFALFA AND RYE

Date.	Days.	Number of microorganisms per gram of silage.					
		Total.	Total acid producers.	Bulgarian group.	Gelatin liquefiers.	Colon type.	Yeasts.
June 1.....	0	50,000,000	1,000,000	210,000	10,000	100,000	100,000
June 2.....	1	400,000,000	100,000,000	40,000,000	10,000	10,000,000	10,000,000
June 4.....	3	1,900,000,000	1,000,000,000	1,900,000,000	10,000	1,000,000	1,000,000
June 7.....	6	300,000,000	10,000,000,000	270,000,000	10,000	1,000	0
June 9.....	8	60,000,000	100,000,000	50,000,000	100,000	0	0
June 11.....	10	240,000,000	100,000,000	60,000,000	10,000	100	0
June 14.....	13	45,000,000	10,000,000	10,000,000	800,000	100	0
June 18.....	17	20,000,000	10,000,000	10,000,000	1,000	10	0
July 2.....	31	15,000,000	100,000,000	5,000,000	10,000	0	1,000
August 6...	66	10,000,000	1,000,000	1,000,000	100,000	0	1,000
August 28..	88	2,000,000	1,000,000	100,000	1,000	100	100
October 16..	137	3,000,000	1,000,000	250,000	10,000	0	0
December 20	202	2,000,000	1,000,000	200,000	100,000	100	1,000

RYE

June 1.....	0	30,000,000	10,000,000	20,000	10,000	1,000	100,000
June 2.....	1	160,000,000	100,000,000	3,000,000	10,000	1,000,000	10,000,000
June 4.....	3	1,000,000,000	100,000,000	900,000,000	1,000	10,000,000	10,000,000
June 7.....	6	600,000,000	10,000,000,000	640,000,000	10,000	100	0
June 9.....	8	80,000,000	100,000,000	80,000,000	1,000	0	0
June 11.....	10	70,000,000	100,000,000	70,000,000	10,000	0	0
June 14.....	13	20,000,000	10,000,000	20,000,000	.....	0	0
June 18.....	17	7,000,000	1,000,000	3,000,000	100,000	0	100
July 2.....	31	15,000,000	1,000,000,000	5,000,000	1,000	0	10,000
August 6...	66	400,000	10,000	300,000	100,000	0	0
August 28..	88	100,000,000	10,000,000	14,000,000	10,000	100	10,000
October 16..	137	20,000,000	1,000,000,000	600,000	1,000	1,000,000	1,000,000
December 20	202	90,000,000	100,000	800,000	1,000,000	100,000	100,000

The microbial curve of development is similar to that noted in 1914, but is more pronounced and conforms more nearly to the results one may expect from normal-silage fermentation. The variations which occurred are attributed to the experimental error of the method of sampling.

No apparent differences were found that will account for the variations in the quality of silage. Stained preparations, made direct from the silage, appeared to agree with the cultural findings. In fact, if the smears were used as a basis of judging the quality of the silage, all would be considered high grade.

The offensive odors characteristic of the alfalfa silage is attributed to protein digestion. The number of gelatin liquefiers were determined in in each silo, with the hope of showing a possible difference between the types of silage. Some of the silage exhibited a slight increase in gelatin liquefiers, but this appears to be of little importance. In all probability the majority of liquefiers are spore-producing types which find unfavorable conditions for growth in the silage. Their numbers in all the silage remained fairly constant throughout the experiment. The slight increase noted in some cases is attributed to experimental error rather than to any actual increase of this type of organism.

The results obtained from these two years' investigations indicate that alfalfa when siloed alone undergoes a typical silage fermentation but that the final product is of a very poor quality. The fermentation, so far as has been observed, is caused by a microbial flora, practically identical with that obtained from silage made from the common forage crops.

However, when a fermentable carbohydrate supplement, such as molasses, corn chop, or sweet sorghum is added to alfalfa at time of siloing, a good quality of silage is produced. Little difference was noted between the microbial flora of such silage and that made from alfalfa alone. The chemical results indicate that more acid is produced in the silage containing the carbohydrate supplements than in that from alfalfa alone. The chemical and bacteriological data fail to offer sufficient evidence to account for the physical differences noted between the two types of silage.

The offensive odors noted in the alfalfa silage are no doubt the result of protein decomposition. By the addition of available carbohydrates this decomposition is checked, and a good quality of silage is the result.

EFFECT OF CARBOHYDRATES ON THE QUALITY OF ALFALFA SILAGE

Lipman and his associates (*13*, *14*) have demonstrated that utilizable carbohydrates when added to soil will hinder ammonification. Kendall and his coworkers (*7–10*), in studying the metabolism of bacteria, have likewise noted the protein-sparing effect of carbohydrates. That fermentation precedes putrefaction, when organisms are grown in media containing both protein and carbohydrates, has also been observed by Kligler (*11*) and Waksman (*18*).

Experiments were conducted for the purpose of observing under laboratory conditions the effect of carbohydrates upon alfalfa-silage fermentation.

Green and cured alfalfa was siloed in sterile milk bottles in the laboratory, with and without carbohydrates. The bottles were hermetically sealed and placed in the dark at room temperature. In those cases where cured alfalfa was used sufficient water was added to make up the proper moisture content. Upon opening the bottles the entire contents of each were finely ground by running through a sterile meat grinder, care being taken to handle as aseptically as possible.

Twenty gm. of this ground forage were placed in a 200-cc. sterile water blank, and from this the required dilutions for the bacteriological analysis were made.

For the chemical analysis 100 gm. of the ground forage were shaken in 1,000 cc. of distilled water for one hour. This was filtered, and an aliquot part of the filtrate used for the various chemical examinations. Twenty gm. of the ground forage were used for moisture determination.

The bacteriological analysis varied slightly in the different experiments, but the technic employed is the same as reported in the previous work. Except in some cases, casein agar was used for the determination of casein digesters, and all gelatin cultures were incubated for 10 instead of 5 days. The chemical analyses included moisture, acidity, amino nitrogen, and ammonia determinations. The acidity readings are expressed in number of cubic centimeters of *N/20* sodium hydroxid required to neutralize 100 gm. of silage. The amino nitrogen was determined by Van Slyke's method, and recorded as milligrams per 100 gm. of silage. The ammonia was determined by direct colorimetric readings of extracts of the ground forage and recorded as milligrams per 100 gm. of silage. All data are calculated upon a moisture-free basis. Three series of experiments were conducted, the first beginning on June 8, 1917, the second on June 25, and the third on July 10.

In the first series 15 milk bottles were filled with alfalfa and 15 with alfalfa plus 5 per cent of cane sugar added as a supplement. The first cutting of alfalfa was used in both cases. The results are given in Table III, first series.

The following notes pertaining to the aroma of the silage were recorded as the bottles were opened for analyses:

June 13. Alfalfa alone, odor good; alfalfa and cane sugar, odor good.
June 15. Alfalfa alone, odor good; alfalfa and cane sugar, odor good.
June 18. Alfalfa alone, odor not so good; alfalfa and cane sugar, odor good.
June 20. Alfalfa alone, odor not so good; alfalfa and cane sugar, odor good.
June 22. Alfalfa alone, undesirable odor; alfalfa and cane sugar, odor good.
September 5. Alfalfa alone, undesirable odor; alfalfa and cane sugar, odor good.
November 20. Alfalfa alone, undesirable odor; alfalfa and cane sugar, odor good.

The alfalfa silage at the conclusion of the experiment had the characteristic offensive odor which had been observed from the alfalfa silage made in the wooden silos. The alfalfa to which 5 per cent of cane sugar had been added produced a good quality of silage with a pleasant sour aroma.

The data submitted in Table III, first series, demonstrate that—

(1) There was little difference in the microbial flora of the two types of sileage.

(2) Both types exhibited a normal microbial curve of development—namely, a rapid increase of numbers for the first week, followed by a decrease.

(3) The acidophylic organisms are the predominating group.

(4) The gelatin liquefiers were inactive in the fermentation.

(5) The acidity produced was characteristic of alfalfa silage. The silage produced from alfalfa with a carbohydrate supplement contained a higher acid content than the alfalfa alone.

TABLE III.—*Effect of carbohydrate upon fermentation of alfalfa silage*

FIRST SERIES, JUNE 8

Days.	Silage.	Bacteriological analysis.						Chemical analysis.		
		Total number of bacteria per gram.	Number of Bulgarian type per gram.	Number of casein digesters per gram.	Number of total acid producers per gram.	Number of gelatin liquefiers per gram.	Percentage of moisture.	Quantity of N/20 sodium hydroxid per 100 gm. of silage.	Quantity of amino nitrogen per 100 gm. of silage.	Quantity of ammonia per 100 gm. of silage.
								cc.	*Mgm.*	*Mgm.*
0	Alfalfa alone	225,000,000	10,000		1,000,000	1,000,000	73.5	236.17	197.43	
0	Alfalfa and sugar	3,000,000	10,000		10,000,000	500,000	69.5	235.29	207.49	
3	Alfalfa alone	3,000,000,000	160,000,000		1,000,000,000	10,000	66.6	725.23	358.17	
3	Alfalfa and sugar	2,500,000,000	1,300,000,000		100,000,000	50,000	70.0	684.80	338.12	
5	Alfalfa alone	3,000,000,000	1,500,000,000		1,000,000,000	50,000	74.0	730.70	367.30	
5	Alfalfa and sugar	2,000,000,000	1,000,000,000		1,000,000,000	1,000	72.5	793.65	360.19	
7	Alfalfa alone	2,000,000,000	600,000,000		10,000,000,000	1,000	75.0	752.70	367.95	
7	Alfalfa and sugar	3,000,000,000	2,000,000,000		10,000,000,000	100	72.5	858.90	393.37	
10	Alfalfa alone	700,000,000	500,000,000		1,000,000,000	1,000	72.5	686.40	405.22	
10	Alfalfa and sugar	250,000,000	300,000,000		1,000,000,000	1,000	70.0	898.80	319.93	
13	Alfalfa alone	400,000,000	500,000,000		10,000,000	1,000	72.5	643.50	475.93	
13	Alfalfa and sugar	400,000,000	200,000,000		100,000,000	1,000	70.0	1,005.80	346.68	
14	Alfalfa alone	225,000,000	7,000,000		50,000,000	1,000	75.0	731.00	442.90	
14	Alfalfa and sugar	200,000,000	14,000,000			1,000	70.0	920.20	326.23	
25	Alfalfa alone	75,000,000	25,000,000		10,000,000	1,000	75.0	688.00	466.55	
25	Alfalfa and sugar	3,000,000	1,000,000		1,000,000	1,000	72.5	922.35	351.52	
33	Alfalfa alone	150,000,000	130,000,000		10,000,000	1,000	75.0	1,112.80	540.72	
33	Alfalfa and sugar	100,000	10,000		10,000	1,000	72.5	1,201.28	383.77	
54	Alfalfa alone	12,000,000	200,000		1,000,000	100,000	70.0	1,003.10	503.97	
54	Alfalfa and sugar	100,000	100		10,000	10,000	80.0	1,166.40	368.28	
89	Alfalfa alone	20,000,000	8,000,000		10,000,000		77.5	721.50	546.72	
89	Alfalfa and sugar	5,000	2,000,000		10,000,000		67.5	918.00	362.32	
165	Alfalfa alone	30,000,000	1,000,000			1,000	75.0	602.00	516.64	
165	Alfalfa and sugar	80,000	2,000,000			1,000	70.0	898.80	399.96	

SECOND SERIES, JUNE 25

0	Alfalfa alone	3,000,000			50,000	10,000	72.5	257.40	199.35				
0	Alfalfa and sugar	700,000			1,000,000	10,000	62.5	297.50	201.28				
2	Alfalfa alone	400,000,000			1,000,000	100	70.0	569.86	291.11				
2	Alfalfa and sugar	80,000,000			10,000,000	1,000	72.5	554.00	243.19				
4	Alfalfa alone	300,000,000			10,000,000	100	72.5	622.00	230.74				
4	Alfalfa and sugar	300,000,000			100,000,000	2,000	70.0	813.20	242.89				
7	Alfalfa alone	200,000,000			100,000,000	100	70.0	535.80	348.82				
7	Alfalfa and sugar	100,000,000			10,000,000	1,000	72.5	664.80	286.34				
11	Alfalfa alone	25,000,000			100,000,000	100	70.0	707.80	358.34				
11	Alfalfa and sugar	35,000,000			1,000,000,000	100	75.0	1,010.00	281.65				
14	Alfalfa alone	18,000,000			2,000,000,000	100	70.0	854.60	313.14				
14	Alfalfa and sugar	10,000,000			100,000,000	100	67.5	854.00	273.15				
26	Alfalfa alone	40,000,000			1,000,000	1,000	68.0	743.80	341.96				
26	Alfalfa and sugar	25,000,000			10,000,000	1,000	72.5	843.80	318.26				
72	Alfalfa alone	2,000,000			50,000	1,000	68.0	618.40	368.90				
72	Alfalfa and sugar	1,000,000					55.0	825.90	296.69				
148	Alfalfa alone	80,000,000				1,000	75.0	410.00	493.70				
148	Alfalfa and sugar	20,000				10,000	70.0	749.00	315.79				

THIRD SERIES, JULY 10

0	Alfalfa alone	60,000,000	20,000	4,000,000	1,000,000	100,000	72.5	345.00	164.01	22.41			
0	Alfalfa and sugar	17,000	3,000,000	10,000,000	10,000	72.5	538.65	34.64					
2	Alfalfa alone	500,000,000	100,000	1,000,000,000	100,000	72.5	664.00	85.45					
2	Alfalfa and sugar	3,000,000,000	20,000	100,000,000	10,000,000	72.5	854.00	62.93					
6	Alfalfa alone	400,000,000	3,000,000	10,000,000	100,000	74.0	710.30	147.13					
6	Alfalfa and sugar	800,000,000		100,000,000	1,000	69.0	903.10	85.87					
8	Alfalfa alone	800,000,000	2,000,000	100,000,000	100,000	72.5	903.60	124.08					
8	Alfalfa and sugar	1,000,000,000	15,000	10,000,000	10,000	70.0	830.60	84.42					
16	Alfalfa alone	75,000,000	15,000	10,000,000	100,000	72.5	750.70	261.83					
16	Alfalfa and sugar	75,000,000	100,000	10,000,000	10,000	70.0	877.40	86.38					
32	Alfalfa alone	30,000,000	200,000	1,000,000	100,000	75.0	858.00	403.13					
32	Alfalfa and sugar	15,000,000	30,000	50,000	10,000	70.0	856.00	298.62					
57	Alfalfa alone	18,000,000	1,000,000	13,000	50,000	75.0	645.00	525.35					
57	Alfalfa and sugar	6,000,000	1,000,000	14,000	50,000	75.0	817.00	350.62					
134	Alfalfa alone	20,000,000	2,000,000	100,000		500,000	72.5	856.00	491.19				
134	Alfalfa and sugar	2,000,000	20,000,000	600,000		500,000	75.0	886.80	379.07				

(6) More amino nitrogen was formed in the alfalfa silage than in the alfalfa and carbohydrate silage. This difference is clearly shown in figure 1.

(7) Carbohydrate supplements exert a protein-sparing effect.

In the second series of experiments begun on June 25, 10 milk bottles were filled with cured alfalfa and the same number with cured alfalfa plus 5 per cent of cane sugar. The alfalfa used was of the first cutting, having been cut on June 8. The data are presented in Table III, second series.

In the second series it was observed that alfalfa alone did not produce undesirable odors as rapidly as in the first series. The offensive odor was in evidence, however, on September 4, 72 days after siloing. The alfalfa with the supplement had a good odor until the series was discontinued, November 20, a period of 148 days.

A study of the data in Table III, second series, reveals the same general conclusion as observed in the preceding experiment. Curves showing the amino-nitrogen determinations of this series are shown in figure 2.

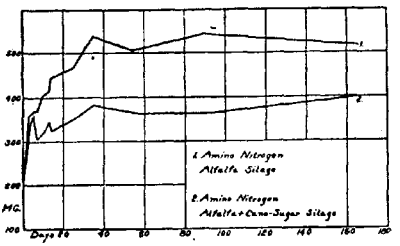

FIG. 1.—Graphs showing protein-sparing effect of carbohydrate in alfalfa-silage fermentation, first series.

The third series of experiments were conducted with the second cutting of alfalfa with and without cane sugar as in the previous experiments. The results are given in Table III, third series.

The quality of the silage as determined by odor was as follows:

July 16. Alfalfa alone, odor slightly undesirable; alfalfa and cane sugar, odor good.
July 26. Alfalfa alone, odor undesirable; alfalfa and cane sugar, odor good.
August 11. Alfalfa alone, odor undesirable; alfalfa and cane sugar, odor good.
September 5. Alfalfa alone, odor undesirable; alfalfa and cane sugar, odor good.
November 20. Alfalfa alone, odor very undesirable; alfalfa and cane sugar, odor good

The results presented in Table III, third series, are very similar to those of preceding experiments. In this series casein agar was used for the determination of casein digesters. The data obtained from this medium are in most respects very similar to those obtained from the use of gelatin. The ammonia determinations indicate the sparing effect of sugar upon the protein, as also do the amino-nitrogen determinations. The ammonia and amino-nitrogen determinations are plotted as curves in figure 3. They indicate the same general results as the curves plotted from the previous data.

It thus appears that physical and chemical differences between the two types of alfalfa silage were established. The data further demonstrate that the carbohydrate acts as a protein sparer.

The problem was discontinued at this point on account of the author's entering other work for the period of the war. As a result, the causative agents of the protein decomposition were not determined. However, the following suggestions pertaining to this question are offered:

It is recognized that the decomposition must be the result of microbial or plant-enzym activity or their associative actions. Previous publications issued from this laboratory mention the importance of microorganisms in silage fermentation (*5*, *6*). From these results it is concluded that the major fermentation processes in silage ripening are the result of microorganisms. That plant enzyms are active is not doubted, but their activities are of minor importance. Lamb in a recent paper on corn-silage fermentation (*12*) concludes (a) that bacteria are mainly responsible for the acid production and the disappearance of sugar; (b) that alcohol results from enzym activity first, but later by yeast fermentation; (c) that protein decomposition is caused by enzyms first, and later by microorganisms; and (d) that carbon dioxid is liberated by enzymic or respiratory changes primarily, but that yeasts are influential after the first day or two.

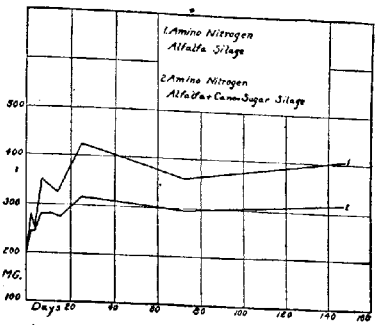

FIG. 2.—Graphs showing protein sparing effect of carbohydrate in alfalfa-silage fermentation, second series.

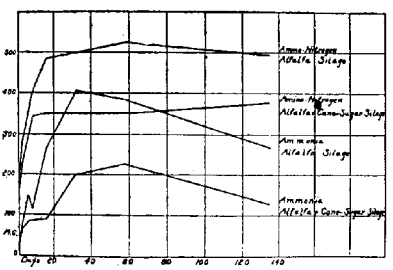

FIG. 3.—Graphs showing protein-sparing effect of carbohydrate in alfalfa-silage fermentation, third series.

Observations of silage made under laboratory conditions at this Station indicate that yeasts are the primary factors of alcohol and carbon-dioxid production. In the study of the microbial flora of silage it was noticed that as a rule the curve of yeast development indicated a very rapid increase of yeast cells within the first two or three days followed by a rapid to gradual decrease. It was further noticed that during the period between 12 and 72 hours after siloing the material underwent a violent fermentation, with the liberation of large amounts of carbon dioxid. During this early fermentation considerable

difficulty occurred in keeping the bottles sealed. This was finally accomplished by wiring the rubber stoppers and sealing with paraffin. That this fermentation was quite active was likewise noted by the bursting of several of the stoppered bottles during this stage. Forage siloed in the presence of chloroform failed to show this characteristic fermentation; nor did the material when opened indicate any signs of fermentation common to silage.

It is a natural assumption that yeasts would reach their maximum numbers and thereby cause an active fermentation in the early period of siloing. This active growth continues until the free oxygen has been consumed. At this stage their growth will be retarded somewhat by the anaerobic conditions produced, and finally checked by the acid fermentation. As a result of this yeast fermentation more or less alcohol is produced, but on account of the presence of the oxygen incorporated in the material when siloed, active growth development is stimulated probably more than alcoholic fermentation. The slow accumulation of alcohol may result from the small number of yeast cells, which persist in the silage for some time, or to zymase liberated from the degenerated yeast cells, or from plant enzyms.

Sherman and Bechdel (*16*), in a late publication pretaining to cornstover silage give as their opinion that the rôle of microorganisms is not as important as that of plant cells. The data offered are too meager and of such a character as not to justify their conclusion.

It is entirely plausible that plant enzyms may cause the protein decomposition noticed in the alfalfa silage. In view of the fact, however, that no such decomposition was observed in alfalfa siloed with chloroform and that chloroform is supposed to offer little injurious action upon such types of proteolytic enzyms, it appears that the cause it to be looked for elsewhere.

It is suggested that at least a small part of this proteolytic action may result from the ability of the acid producers to utilize protein as a source of energy in the absence of available carbohydrates. This digestive effect of the lactic acid bacilli has been demonstrated by Bertrand and Weisweiller (*1*), and Heinemann and Hefferan (*4*). Likewise, Hastings and his coworkers (*3*) show that from the analyses of pure lactic acid milk cultures, the percentage increase of soluble nitrogen varied from 12.5 to 62.5. Hart, Hastings, Flint, and Evans (*2*) have demonstrated the ability of *Bacillus casei* to produce ammonia. Hastings found large numbers of the *B. bulgaricus* group in chedder cheese, and concluded that, since they develop after the fermentation of the sugar, they must have some other source of carbon and energy than milk sugar.

No study of the anaerobic flora of the silage was made, but it would not be surprising to establish some proteolytic action with an anaerobic flora.

CONCLUSIONS

(1) Silage made from alfalfa alone is of an inferior quality. By the addition of an available carbohydrate supplement, a good quality of silage may be produced.

(2) The microbial flora of the two types of silage, so far as noted, was practically identical.

(3) The chemical data demonstrate that alfalfa with a supplement produced a higher acid content than alfalfa alone.

(4) Alfalfa silage shows a greater amount of protein decomposition, as evidenced by the amino-nitrogen and ammonia determinations.

(5) The decomposition of protein was checked by the addition of a utilizable carbohydrate.

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BROWN CANKER OF ROSES, CAUSED BY DIAPORTHE UMBRINA

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INTRODUCTION

In April, 1917, an apparently unreported fungus disease producing a canker on stems of rose (*Rosa* spp.) was brought to the attention of the Office of Pathological Collections. In the National Rose Test Garden at the Arlington Experimental Farm, Virginia, where over 900 named species and horticultural varieties of roses are grown, nearly all were affected. All the brier roses, the rugosas, the moss roses, some of the ramblers, and most of the named species were unaffected. The canes had been badly injured the previous season by a severe hailstorm, and the disease in some cases had gained entrance through these wounds, although there were many instances of infection where external injury could not be detected. The cankered canes were cut out and burned, and to all appearances the new growth was healthy. When observed in March, 1918, although there was no evidence of hail injury, the canker was again equally as prevalent as in 1917, thereby necessitating the severe cutting back of the plants.

The disease appears to be more or less widely distributed, specimens having been received from Georgia, Virginia, West Virginia, and the District of Columbia. The dates of these collections range from 1903 to 1918. In June, 1918, Dr. L. M. Massey, Plant Pathologist at Cornell University, who has given special attention to rose diseases, forwarded to this laboratory a culture made from rose material collected in Connecticut. A comparison of this culture with the fungus isolated by the writer showed that the two organisms are identical

CHARACTER OF THE DISEASE

This canker may occur on any portion of the cane, diseased areas being raw-umber in color, sometimes surrounded by a purple border (Pl. 46, B). The cankered surface is dotted with fruiting bodies, the pycnidial and ascosporic stages of the parasite. In mature specimens the small protruding beaks of the perithecia may be seen. The margin of a canker is generally defined only by the difference in color between the normal and cankered portions, and to this contrast in color is due the conspicuous and disfiguring effect of the disease (Pl. D). The canker differs in color and appearance, from the common canker caused by *Coniothyrium fuckelii* Sacc. In color the latter canker is a Roman sepia or dirty

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umber. Its lesions are often depressed with raised margins, and the pycnidia in macroscopical appearance are darker than the fruiting bodies of the brown canker (Pl. 46, A).

ISOLATION OF THE CAUSAL ORGANISM

Following Keitt's method[1] for obtaining pure-culture strains, the writer made single pycnospore and single ascus isolations. It was thought advisable to isolate a single ascus rather than an ascospore, as the ascospores, especially when immature, appear much the same in in size and color as the pycnospores. From these isolations of the fungus transfers were made to various media. From the imperfect-stage strain only pycnidia developed, while from the perfect-stage strain growing on sterilized rose canes the formation of pycnidia was followed by the development of a perfect stage similar to that found in nature. The growth from the pycnidial stage was labeled "A," and that from the perithecial stage "B." Cultures of the two stages were used in inoculation experiments.

INOCULATION EXPERIMENTS

Inoculation tests were made on rose plants under ordinary greenhouse conditions and on cut rose canes kept in a moist atmosphere under bell jars in the laboratory. The inoculations were made by smearing spores or a portion of the culture over an incision in the cane made with a sterile scalpel. The incision was then covered with sterile cotton moistened with sterile water.

Inoculations with stage A in the greenhouse produced practically no infection. On one plant, which was inoculated on May 28, 1917, and placed under a bell jar, and which had gradually lost most of its leaves from mildew, the disease developed after a long period, producing the pycnidial stage of the fungus. Freshly cut rose canes placed under bell jars became infected in a few days. On stems inoculated on February 8 the disease had progressed upward 4 inches by February 20; and by February 23 it had progressed to the tip of the cane and had passed downward from the point of inoculation a distance of 4 inches, darkening and killing the stem. The cane was somewhat lighter in color for about 1 inch above and ½ inch below the point of inoculation. In this region were developed the characteristic pycnidia from which spore tendrils were exuded. The controls subjected to similar treatment remained fresh and healthy, and there was no discoloration of the tissue near the point of inoculation.

Inoculations were made with B in the greenhouse on October 20. In four days there was slight infection. The plants were kept under observation until January, but the area of infection remained very

[1] KEITT, G. W. SIMPLE TECHNIQUE FOR ISOLATING SINGLE-SPORE STRAINS OF CERTAIN TYPES OF FUNGI. *In* Phytopathology, v. 5, no. 5, p. 266-269, 1 fig. 1915.

limited, the cane being darkened and killed for a radius of about ¼ inch from the point of inoculation. A microscopic examination revealed the presence of hyphæ in the darkened tissue, but the fungus did not fruit on the diseased area. Plantings were made in corn-meal agar poured plates from small portions cut from the edge of the discolored tissue with a sterile scalpel, then immersed in a mercuric-chlorid solution (1:1,000) for about three minutes, and rinsed in sterile water. In such cultures made on January 19 the imperfect stage developed in five days, while cultures made similarly from the controls remained sterile.

Inoculations with B were again made in the greenhouse on January 30. No infection resulted from two of the inoculations, which may not have been kept sufficiently moist, but on February 13 it was observed that the third inoculation had produced infection. Plantings were made in April from the discolored region and from a corresponding region of the control. The fungus was reisolated from the inoculated plant, while no growth appeared in the culture made from the control (Pl. 47, C, D).

On January 17 freshly cut rose canes were placed under the bell jars in the laboratory and inoculated with B. Infection appeared in 10 to 15 days from all of the inoculations. The progress of the disease may be described from the observations taken on one of the inoculated canes, which when first observed was girdled and darkened for 1 inch above the point of inoculation. In two days the disease advanced upward 3 inches, and in three days the entire cane above the point of inoculation was affected. The disease passed downward less rapidly. An area, lighter in color and similar to that described in the A inoculations, appeared near the point of inoculation. In this area the typical pycnidia developed (Pl. 47, A, B).

Two inoculations were made by smearing an infusion of spores of stage B on the leaf buds. From each inoculation infection appeared at the base of the bud in about 10 days, and the disease advanced for several inches along the stem. The spores evidently germinated on the leaf tissue, the fungus passing through the bud into the cane. This experiment does not indicate that the fungus may gain entrance through the healthy or uninjured buds, for in making the inoculations no precaution was taken to avoid injuring the tender leaflets.

These experiments [1] establish the pathogenicity of the fungus. The percentage of infection appears to vary with temperature and humidity as shown by the results of the different series of inoculations. Inoculations made under bell jars gave 100 per cent infection, while those made without covering the plants gave a smaller percentage of infection. When the fungus is once established, it advances rapidly, producing the characteristic lesions.

[1] Subsequent to the writing of this paper, the information has been received from Dr. L. M. Massey, of Cornell University, that he has also made successful inoculations on roses in the greenhouse with what appears to be the same fungus.

DESCRIPTION OF THE FUNGUS

From a study of the fungi reported on the rose no description has been found that seems to apply to this organism. The characters of the ascogenous stage, both in its development in nature and in culture, place it most nearly in the genus Diaporthe. It differs from that genus in having continuous ascospores which may, however, become pseudo-septate. The spores of the genus Diaporthe are typically 2-celled, but in some species they are described as nucleate, 1-celled while young, or obscurely septate. The imperfect stage appears to be most closely related to the genus Phomopsis. In view of the above considerations, it has seemed that for the present the fungus may best be referred to the genus Diaporthe and will be described as *Diaporthe umbrina*.

Diaporthe umbrina, n. sp.[1]

Pycnidia subglobose to lens-shaped, walls generally rather thin, thickened at apex, embedded, rupturing the epidermis, irregularly ostiolate, simple or chambered, 200 to 300 μ in diameter; pycnospores subfusoid, straight or slightly curved, hyalin, 4.8 to 11.2 by 2 to 3.2 μ; sporophores simple or branched, tapering above, 12 to 40 μ in length.

Perithecia membranaceous, two to five, immersed in a valsoid stroma around pycnidium, globose, with beak scarcely projecting above the epidermis, 100 to 290 μ in diameter; beaks 150 to 195 μ in length; asci clavate, subsessile, aparaphysate, 30 to 50 by 6.4 to 8 μ; spores elliptical, usually hyalin, when mature sometimes light olivaceous, continuous or sometimes with one pseudo-septum, 8 to 11.2 by 3.2 to 4 μ.

Forms cankers on the stems of cultivated roses.

Type material collected at Arlington Experimental Farm, Virginia, April, 1917. Specimens deposited in the herbarium of the Office of Pathological Collections, Bureau of Plant Industry, United States Department of Agriculture, Washington, D. C.

In figure 1 is shown a pycnidium as it occurs in nature. The basal wall of the pycnidium is often very much thickened near the center, projecting upward in the cavity of the pycnidium. Protrusions may develop from other portions of the wall, forming a chambered pycnidium as illustrated in figure 1, *a*. The parenchymatous upper portion of the pycnidium is composed of small, closely packed, thin-walled cells. The fungus suggests somewhat the appearance of a species of Myxosporium in certain sections through the pycnidium in which the thickened upper portion has apparently fallen away. Great variability was apparent in the development of the pycnidial stage in culture. It was observed that on prune-agar poured plates made from stage A that the spores were first borne directly from the mycelium and that an abundance of

[1] **Diaporthe umbrina,** sp. nov.—Pycnidiis subglobosis vel lenticularibus, parietibus plerumque aliquid tenuibus, apice incrassatis, immersis, epidermidem rumpentibus, irregulariter ostiolatis, simplicibus vel loculatis, 200–300 μ diameter pycnosporis subfusoideis, rectis vel curvulis, hyalinis, 4.8–11.2×2–3.2 μ, sporophoris simplicibus vel ramosis, attenuatis, 12–40 μ longis; peritheciis membranaceis, 2–5, valsea stromate circa pycnidium immersis, globosis, rostratis, 100–290 μ diameter; rostris saepe supra epidermidem prominentibus, 150–195 μ longis; ascis clavatis, subsessilibus, aparaphysatis, 30–50×6.4–8 μ; sporis ellipticis, plerumque hyalinis, maturis interdum pallide olivaceis, continuis vel interdum 1-pseudo-septatis, 8–11.2×3.2–4 μ.

Cancros in sarmentis Rosarum cultarum formans, Arlington, Virginia.

thin-walled membranous pycnidia soon developed. The fungus was transferred to the rose-stem medium, where the pycnidial development was similar to that in nature, the pycnidia being simple or somewhat chambered, 140 to 375 μ in diameter with the walls in the upper portion somewhat more stromatic. The perithecia develop in the stromatic growth below the pycnidia which in culture become crumpent. Plate 46, C, shows an enlarged portion of a culture, with protruding beaks of the perithecia. In culture there may be as many as eight perithecia in a stroma, measuring 290 to 400 μ, with beaks 250 to 500 μ in length. Figure 2, *a*, shows a vertical section of perithecia in nature. It will be observed from this illustration that in nature the beaks of the perithecia which extend toward the pycnidial rupture in the epidermis do not protrude proportionally as far as they do in culture. In figure 3 is shown the arrangement of the perithecia and pycnidia as they occur in nature.

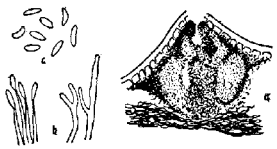

FIG. 1.—*Diaporthe umbrina: a*, Vertical section of a pycnidium in nature, × 80; *b*, simple and branched sporophores; *c*, pycnospores, × 360.

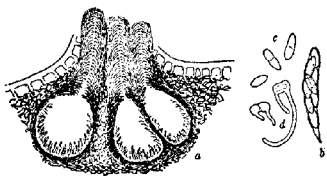

FIG. 2.—*Diaporthe umbrina: a*, Vertical section of perithecia in nature, × 80; *b*, an ascus; *c*, ascospores; *d*, germinating ascospores, × 420.

The development of pycnospores on the host and in culture is similar. The filiform spores which sometimes develop in species of Phomopsis in addition to the typical fusoid spores have not been observed. The pycnospores germinate readily, producing one or two germ tubes. The sporophores, which are sometimes branched, are variable in length, but generally measure from 12 to 20 μ. The base of the sporophore is often broad and irregular in shape, becoming very slender above (fig. 1, *b*). The ascospores in nature are most often continuous, but they may be 2-guttulate or 1-pseudoseptate. Upon germination the spores become 2-guttulate or pseudoseptate and may be slightly constricted at the center, and one germ tube is produced toward each end of the spore (fig. 2, *b*, *c*, *d*).

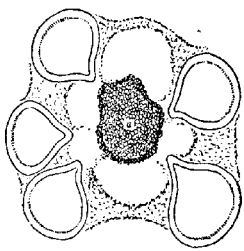

FIG. 3.—*Diaporthe umbrina:* Tangential section showing relative arrangement of pycnidium and perithecia in nature, a pycnidium surrounded by five perithecia; *a*, proliferous stratum extending upward from base of pycnidium.

CULTURAL CHARACTERS

The organism was grown on steamed corn meal, corn-meal agar, oat agar, prune agar, and rose canes. Both strains of the fungus grow rapidly on these media, producing the imperfect stage in three or four days with very little superficial mycelial growth. The pycnidia are developed in great abundance, extruding cinnamon-buff[1] spore masses, which often cover the entire surface of the medium.

Plate 46, D, shows a B culture on rose canes after 22 days' growth, while Plate 46, C and E, reproduces photomicrographs of a similar culture after three months, showing the extruding pycnospore masses and the beaks of the perithecia. Only cultures on rose stems in test-tubes containing a very small amount of moisture and kept approximately at a temperature of 17° C. developed the perfect stage of the fungus. On steamed corn-meal media the superficial mycelial growth was most abundant, appearing pure white at first and soon becoming gray. On prune agar and on corn-meal agar poured plates the culture develops radially from the point of inoculation, concentric rings of the pycnidia appearing at more or less regular intervals.

LIFE HISTORY OF THE FUNGUS

That the life cycle of the fungus may be completed in a comparatively short time is shown from the cultural experiments and from the field observations. From the inoculation experiments it has been shown that infection occurs from both pycnospores and ascospores and that lesions may be produced in from 4 to 15 days. A culture made on February 11 from pycnospores produced from stage B had developed the two stages by April 15. On field material collected on March 18 the imperfect stage was present, together with the immature perfect stage. In May spores of both stages germinated in culture, producing the perfect and imperfect stages as previously described, indicating that a period of rest is not essential for the completion of the life cycle. On the other hand, the spores probably remain viable for a long time, since pycnospores from very thin poured-plate cultures which had dried were viable after having been kept for four months at a temperature of about 17° C. How the fungus overwinters and the manner in which the first infections are produced are still subjects of investigation. It is very probable that the ascospores live over a winter in the old canker and produce the early spring infections. It is not known how the spores are disseminated, whether by wind, rain, insects, or on cultural implements, but it is probable that rain plays an important part in distributing the exuded pycnospores.

CONTROL MEASURES

Preliminary experiments in controlling the disease by cutting out and burning the cankered stems have been made, but the results during the

[1] RIDGWAY, Robert. COLOR STANDARDS AND COLOR NOMENCLATURE. 43 P., 53 col. pl. Washington, D. C., 1912.

single year that the disease has been under observation were negative. From the present knowledge of this rose canker and its causal fungus, together with the control measures employed for diseases of a similar nature, certain preventive methods are suggested. Rose gardens should be carefully inspected to determine whether the disease is present and precautions taken to prevent the entrance of the fungus into healthy gardens. Nursery stock for planting should be carefully examined, and all cankered plants should be destroyed. If possible, plants should be introduced only from gardens known to be free from the disease. In gardens where the canker is already established, measures leading to the eradication of the fungus should be adopted. Cankered stems should be cut away and burned. It may be advisable to disinfect the cut ends of the stems and to sterilize the pruning implements after each operation. The use of a fungicide to protect the plants from further infection is suggested. In the fall after the plants have become dormant and again early in the spring strong Bordeaux mixture[1] may be applied. It is probably important that the plants be well covered with the fungicide during the early spring months when the lesions first appear and the disease makes rapid progress. Gardens should be carefully watched in the spring in order that stems showing infection may be cut away and not become sources of secondary infection. As blossoming time approaches, ammoniacal copper carbonate may be substituted for the Bordeaux mixture, as it does not discolor the foliage. A complete study of the varieties resistant to this canker has not been made, but, as stated in the introduction of this paper, in the National Rose Test Garden, where most classes of cultivated roses are represented, the brier roses, the rugosa roses, the moss roses, some of the ramblers, and most of the named species are apparently resistant.

SUMMARY

(1) A canker of roses caused by the fungus *Diaporthe umbrina* is probably widely distributed and is known to occur in the District of Columbia, Virginia, West Virginia, Georgia, and Connecticut, having been reported at various times during the past 15 years.

(2) The causal organism produces in cankers on living rose stems a pycnidial and a perfect stage.

(3) In culture both the pycnidial and perithecial stages of the fungus have developed.

(4) The disease has been produced on rose stems from both pycnosporic and ascosporic stages of the fungus.

(5) The control measures suggested are the use of only healthy nursery stock for planting, the removal and burning of diseased canes from affected gardens, and the application of a fungicide in the fall, again in spring before the first symptoms appear, and during the spring when the fungus is active.

[1] For directions for the preparation and application of fungicides for rose diseases see MULFORD, F. L., ROSES FOR THE HOME. U. S. Dept. Agr. Farmers' Bul. 750, 36 p., 27 fig. 1916.

PLATE D

Rose cane showing lesion of the canker caused by *Diaporthe umbrina*.

(600)

PLATE 46

A.—Rose cane showing the appearance of the canker caused by *Coniothyrium fuckelii*.

B.—A rose stem showing local infections produced by *Diaporthe umbrina*.

C.—Culture of *Diaporthe umbrina* from stage B on a rose stem showing beaks of perithecia. × 20.

D.—Culture on a rose stem from stage B showing spore masses extruded from pycnidia.

E.—Pycnospore masses from culture shown in figure C. × 20.

Photographed by Mr. J. M. Shull.


A
B
C
D
E

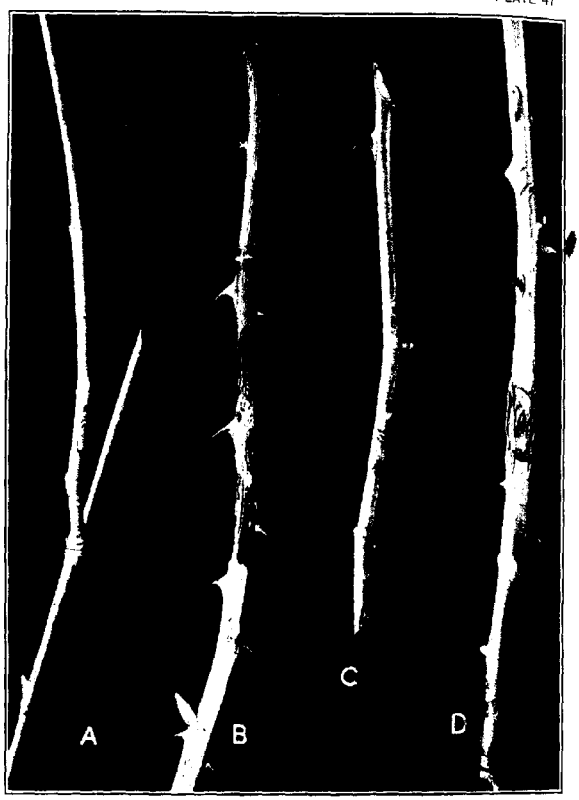
A
B
C
D

PLATE 47

Diaporthe umbrina: Results of inoculations

A.—Control.

B.—Rose stem showing infection produced by inoculation with stage B; cut rose stems placed in moist atmosphere under bell jars in the laboratory.

C.—Control.

D.—Rose stem showing infection produced by inoculation with stage B; rose plants in the greenhouse.

Photographed by Mr. J. M. Shull.

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EFFECT OF CARBON DISULPHID AND TOLUOL UPON NITROGEN-FIXING AND NITRIFYING ORGANISMS

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INTRODUCTION

In previous publications [1] the writer has presented experimental data showing the effect which carbon disulphid and toluol have upon the total number of bacteria (bacteria that would develop upon agar plates) and upon the accumulation of nitrate nitrogen in soils. It is the purpose of this paper to give data showing the effect of similar treatment of soils upon the nitrogen-fixing organisms, together with additiomal data upon nitrification.

The appearance during the past few years of many excellent reviews of the literature relative to the treatment of soils with volatile antiseptics renders a discussion of previous work unnecessary. The reader is referred especially to the review given by Kopeloff and Coleman.[2] It is sufficient to say that the results heretofore reported have been very irregular and inconclusive. It is believed that the data reported in this paper will offer, in part at least, an explanation for some of these irregularities.

EXPERIMENTAL METHODS

In general, the methods used have been similar to those previously employed by the writer. Soils known to possess good nitrogen-fixing and nitrifying powers have been used. Given quantities of soil by weight were treated with varying quantities of carbon disulphid and with toluol. The reagent was then thoroughly mixed in, and the soil immediately placed in an air-tight container and left for three days. At the end of this period those samples from which the reagent was not to be evaporated were made up to the required degree of saturation with sterile water, the rubber stoppers replaced with cotton, and incubated at room temperature. If the antiseptic was to be evaporated, either the containers were left open or the soil was emptied into sterile petri dishes and left until the soil was air-dry and no odor of the chemical remained. Water was then added and the samples incubated at room temperature. At various stages during treatment or during incubation the nitrogen-fixing ability was tested, and the nitrates present in the soil were determined.

[1] Gainey, P. L. The effect of toluol and CS_2 upon the microflora and fauna of the soil. *In* Mo. Bot. Gard. 23d Ann. Rpt., p. 147–169. 1912. Literature, p. 168–169.

——— Effect of CS_2 and toluol upon nitrification. *In* Centbl. Bakt. [etc.]. Abt. 2, Bd. 39, No. 13–25, p. 584–595, 2 fig. 1914.

[2] Kopeloff, Nicholas, and Coleman, D. A. A review of investigations in soil protozoa and soil sterilization. *In* Soil. Sci., 3, no. 3, p. 197–269. Literature cited, p. 248–269.

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Dec. 16, 1918
Key No. Kans.-17

In the nitrogen-fixing experiments 50 cc. of a sterile culture solution in 350-cc. Erlenmeyer Jena flasks were inoculated with 2 or 5 gm. of soil and incubated, usually for three weeks. A medium consisting of the following compounds was used:

Potassium phosphate (Kn_2HPO_4)	0.2 gm.
Magnesium sulphate ($MgSO_4$)	.2 gm.
Sodium chlorid (NaCL)	.5 gm.
Mannit	20.0 gm.
Ferric chlorid ($FeCl_3$)	Trace.
Water	1,000 cc.

This solution was made slightly alkaline to phenolphthalein with sodium hydroxid. In other experiments this medium has never failed to give the characteristic Azotobacter film if the organisms were present in sufficient numbers and other conditions were favorable. The figures reported are in all cases actual gains in nitrogen.

Inoculations were made in duplicate. During incubation the growth of Azotobacter was noted, and at the end of the incubation period the total nitrogen was determined. Only the average of duplicate nitrogen determinations are reported.

In the tables giving results the Azotobacter film has been reported as typical if the characteristic brown or black uniform growth covered the surface. A question mark (?) has been placed opposite those samples from which only one duplicate gave a film or when there were Azotobacter present, but which failed to give the characteristic growth.

Nitrate nitrogen was determined in duplicate by the phenol-disulphonic-acid method. Qualitative tests for ammonia were made with Nessler's reagent. Where the aqueous extract of the soil gave only a slight yellow color, it was reported as a trace. When the color was somewhat more pronounced, it was reported as slight; and where a heavy brick-red precipitate was formed when the reagent was added, it was reported as abundant. In some instances quantitative determinations of ammonia nitrogen were made by distillation with magnesium oxid. A number of determinations upon samples reported as a trace showed from 0.3 to 0.5 mgm. of nitrogen per 100 gm. of soil. Variations from these methods were sometimes made, but they are mentioned in connection with the individual experiment to which they apply.

EXPERIMENTAL DATA

The soil for these experiments was furnished through the kindness of Prof. Walter G. Sackett, of the Colorado Agricultural Experiment Station. It came "from a locality where the niter trouble has been very severe and where nitrification has evidently been very active in the past if not at present." This soil was used because of the exceptionally vigorous nitrogen-fixing (Azotobacter) flora.

Fifty-gm. samples containing 6.5 gm. of water each were treated with varying amounts of toluol and carbon disulphid as indicated in Table I. The soil was then immediately placed in 500-cc. bottles, tightly stoppered, and left for three days. At the end of this time the water content of the nonevaporated samples was made up to 12 cc. or one-half saturation, and the stopper replaced with cotton. The samples were incubated under conditions which would retard loss of moisture and were not opened except when analyses were made. The evaporated samples were treated exactly alike, except that at the end of the first three days the soil was emptied into petri dishes, left thus for 48 hours, replaced in the bottle, and the moisture content made up to one-half saturation. Tests for nitrogen fixation were made at the end of four weeks and again after six months' incubation, and the nitrate content was determined at the end of six months.

Before the six-months' analyses were made the moisture content of all samples had fallen very low, some samples being practically air-dry. This apparently had no effect upon nitrogen-fixing organisms, but did obscure the recovery of active nitrification. In other words, before sufficient time had elapsed for the recovery of active nitrification, the moisture content of many samples had fallen so low that nitrification was impossible. The results are reported in Table I.

There is little difference between the evaporated and nonevaporated samples in the amount of nitrogen fixed; 0.25 cc. of toluol per 100 gm. of soil destroyed the Azotobacter, and there is no evidence of recovery. The same quantity checked nitrification, but the quantity necessary to destroy the nitrifying organisms is very much higher, the samples receiving even 5 cc. having partly recovered after six months. As mentioned above, the failure to recover was probably due to low water content rather than to the toluol. Ammonia accumulated only when nitrification was checked.

The quantity of carbon disulphid necessary to destroy Azotobacter was only 0.1 cc. per 100 gm. of soil, and there is no evidence of recovery even after six months. In the nonevaporated samples, 0.1 cc. checked nitrification, while 0.25 cc. were required to check the process in evaporated samples. There is evidence of recovery of nitrification even with 5.0 cc. of carbon disulphid. Ammonia accumulated only when nitrification was checked.

Even when Azotobacter were destroyed, the ability to fix nitrogen was not destroyed with the largest quantity of carbon disulphid or toluol applied in these experiments. However, fixation by organisms other than Azotobacter appeared to decrease as the application of chemicals increased.

A second set of experiments was conducted, in which the soil had been in the laboratory much longer and was almost air-dry, containing only

4 per cent of water. The treatment of the soil otherwise was essentially the same. After the reagents had acted for three days, the unevaporated samples were made up to one-half saturation. Those from which the reagents were evaporated were left open for two days and then made up to the same degree of saturation. All were cotton-plugged and incubated at room temperature. The water lost through evaporation was replaced from time to time.

TABLE I.—*Effect of carbon disulphid and toluol upon nitrogen fixation and nitrate accumulation*

CHEMICALS NOT EVAPORATED

Treatment.	Analyzed after four weeks.			Analyzed after six months.			
	Azotobacter film.	Nitrogen fixed.[a]	NO_3[b]	Azotobacter film.	Nitrogen fixed.[a]	NO_3[b]	NH_3[b] nitrogen.
		Mgm.	*Mgm.*		*Mgm.*	*Mgm.*	
Control, 0 cc	Typical.	10. 40	11. 3	Typical.	9. 12	18. 5	Trace.
Toluol:							
0. 01 cc	...do....	11. 80	12. 0	...do....	9. 70	18. 5	Do.
0. 05 cc	...do....	12. 40	11. 3	...do....	9. 01	18. 5	Do.
0. 10 cc	...do....	11. 75	12. 0	...do....	9. 07	18. 5	Do.
0. 25 cc	None...	7. 05	4. 5	None....	4. 32	20. 0	Do.
0. 50 cc	...do....	10. 55	3. 0	...do....	3. 40	3. 5	Abundant.
1. 00 cc	...do....	7. 05	4. 5	...do....	4. 61	3. 5	Do.
5. 00 cc	...do....	7. 75	3. 6	...do....	5. 27	3. 3	Do.
Control, 0 cc	Typical.	12. 45	12. 8	Typical.	9. 12	18. 5	Trace.
Carbon disulphid:							
0. 01 cc	...do....	12. 05	12. 8	...do....	9. 18	18. 5	Do.
0. 05 cc	...do....	11. 90	12. 8	(?)	7. 45	18. 5	Do.
0. 10 cc	None....	4. 25	13. 8	None....	5. 32	20. 0	Do.
0. 25 cc	...do....	4. 45	3. 2	...do....	3. 13	4. 1	Abundant.
0. 50 cc	...do....	3. 45	2. 2	...do....	0. 55	3. 5	Do.
1. 00 cc	...do....	2. 15	3. 3	...do....	0. 65	3. 3	Do.
5. 00 cc	...do....	3. 15	3. 5	...do....	6. 68	3. 2	Do.

CHEMICALS EVAPORATED

Control 0 cc	Typical.	9. 45	12. 0	Typical.	10. 28	21. 8	Trace.
Toluol:							
0. 01 cc	...do....	9. 20	12. 0	...do....	9. 18	21. 8	Do.
0. 05 cc	...do....	8. 67	10. 9	...do....	10. 28	21. 8	Do.
0. 10 cc	...do....	10. 75	12. 0	...do....	9. 62	20. 0	Do.
0. 25 cc	None....	4. 05	3. 6	None....	5. 00	24. 0	Do.
0. 50 cc	...do....	5. 20	.....	...do....	5. 38	12. 0	Abundant.
1. 00 cc	...do....	5. 05	3. 6	...do....	4. 17	21. 8	Trace.
5. 00 cc	...do....	4. 45	3. 5	...do....	5. 27	6. 7	Abundant.
Control 0cc	Typical.	10. 00	12. 0	Typical.	9. 73	19. 2	Trace.
Carbon disulphid:							
0. 01 cc	...do....	10. 10	12. 0	...do....	9. 73	24. 0	Do.
0. 05 cc	...do....	9. 35	12. 0	(?)	6. 87	21. 8	Do.
0. 10 cc	None....	4. 45	3. 5	None....	4. 12	21. 8	Do.
0. 25 cc	...do....	3. 35	3. 5	...do....	3. 35	6. 3	Abundant.
0. 50 cc	...do....	3. 30	3. 5	...do....	.....	5. 8	Do.
1. 00 cc	...do....	3. 15	3. 5	...do....	2. 74	4. 8	Do.
5. 00 cc	...do....	2. 80	2. 0	...do....	3. 24	12. 0	Do.

[a] ... per 100 gm. of soil.

Cultures for Azotobacter were made from the evaporated samples after the exposure for evaporation and from all cultures after 16 weeks' incubation. Since the principal point to be determined was whether Azotobacter were killed, a total nitrogen determination was not made on the nitrogen-fixing cultures at the 16 weeks' analysis. Also, previous experiments had shown that the quantity of nitrogen fixed when the characteristic film developed did not vary beyond the experimental error and that the quantity fixed in absence of Azotobacter was usually from one-fourth to one-half than when Azotobacter were present. The character of growth was, therefore, a sufficient index for the object in view. The results are presented in Tables II and III.

TABLE II.—*Effect of toluol upon nitrogen fixation and nitrification*

Treatment.	Toluol not evaporated.			Toluol evaporated.				
	Incubation 10 weeks.	Incubation 17 weeks.		No incubation.	Incubation 15 weeks.	Incubation 17 weeks.		
	Azotobacter film.	NO_3.[a]	NH_3 nitrogen.	Azotobacter film.	Nitrogen fixed.[b]	Azotobacter film.	NO_3.[a]	NH_3 nitrogen.
		Mgm.			*Mgm.*		*Mgm.*	
Control, 0 cc....	Typical.	17.5	Trace.	Typical	.....	Typical.	18.0	Trace.
Toluol:								
0.02 cc.....	...do....	18.5	...do....	...do....	8.23	...do....	16.4	Do.
0.10 cc.....	...do....	16.9	...do....	...do....	7.56	...do....	16.9	Do.
0.20 cc.....	...do....	16.4	...do....	...do....	8.34	...do....	18.5	Do.
0.40 cc.....	...do....	19.2	...do....	...do....	9.91	...do....	17.5	Do.
0.60 cc.....	...do....	16.2	...do....	...do....	8.51	...do....	.....	Do.
0.80 cc.....	...do....	16.2	...do....	...do....	8.28	...do....	16.9	Do.
1.00 cc.....	...do....	18.0	...do....	...do....	10.64	...do....	17.5	Do.
1.20 cc.....	...do....	18.0	...do....	...do....	9.30	...do....	17.5	Do.
1.40 cc.....	...do....	16.9	...do....	...do....	8.45	...do....	17.5	Do.
1.60 cc.....	...do....	20.7	...do....	...do....	9.13	...do....	18.0	Do.
1.80 cc.....	...do....	16.9	...do....	...do....	9.63	...do....	17.5	Do.
2.00 cc.....	...do....	19.2	...do....	...do....	8.40	...do....	16.9	Do.
10.00 cc.....	None....	5.8	[c]3.0	...do....	9.02	...do....	18.0	Do.

[a] Nitrates in milligram per 100 gm. of soil.
[b] Nitrogen fixed in milligram per 50 cc. of culture.
[c] Milligrams.

The results presented in Tables II and III are quite different from those given in Table I. For example, the only samples in which Azotobacter were destroyed and in which nitrification was checked were those treated with 10 cc. of either carbon disulphid or toluol. In case of the evaporated samples, even 10 cc. of toluol had no effect. Again, in only those samples in which nitrification was checked was there any accumulation of ammonia.

TABLE III.—*Effect of carbon disulphid upon nitrogen fixation and nitrate accumulation*

Treatment.	Carbon disulphid not evaporated.		Carbon disulphid evaporated.					
	Incubation 16 weeks.	Incubation 17 weeks.	No incubation.			Incubation 16 weeks.	Incubation 17 weeks.	
	Azotobacter film.	NO_3.[a]	NH^3 nitrogen.	Azotobacter film.	Nitrogen fixed.[b]	Azotobacter film.	NO_3.[a]	NH_3 nitrogen.
		Mgm.			*Mgm.*		*Mgm.*	
Control, 0 cc.	Typical.	18.0	Trace.	Typical.	9.18	Typical.	17.0	Trace.
Carbon disulphid:								
0.02 cc	do.	18.0	do.	do.	10.64	do.	20.0	Do.
0.10 cc	do.	18.0	do.	do.	.....	do.	19.4	Do.
0.20 cc	do.	18.0	do.	do.	9.13	do.	19.7	Do.
0.40 cc	do.	17.0	do.	do.	11.03	do.	20.0	Do.
0.60 cc	do.	18.0	do.	do.	.....	do.	17.0	Do.
0.80 cc	do.	17.0	do.	do.	8.25	do.	18.0	Do.
1.00 cc	do.	18.0	do.	do.	.....	do.	18.5	Do.
1.20 cc	do.	17.0	do.	do.	9.91	do.	19.0	Do.
1.40 cc	do.	17.5	do.	do.	8.56	do.	21.1	Do.
1.60 cc	do.	19.4	do.	do.	12.32	do.	20.6	Do.
1.80 cc	do.	19.0	do.	do.	8.45	do.	17.0	Do.
2.00 cc	do.	18.0	do.	do.	9.20	do.	16.3	Do.
10.00 cc	None.	7.8	[c]1.9	None.	−0.84	None.	6.6	[c]2.0

[a] Nitrates in milligrams per 100 gm. of soil.
[b] Nitrogen fixed in milligrams per 50 cc. of culture.
[c] Milligrams.

EFFECT OF MOISTURE CONTENT OF SOIL UPON THE GERMICIDAL ACTION OF CARBON DISULPHID AND TOLUOL

The results obtained from the two sets of experiments given above were so radically different that it seemed necessary to ascertain, if possible, the cause of the differences. The soil, while not the same, was quite similar and it did not seem possible that such wide differences in the effect of the reagents could be due to differences in the character of the soil. An examination of the conditions under which the two sets of experiments were conducted revealed only one important difference. The moisture content of the soil in the first experiments was 13 per cent, while in the second set the moisture content was only 4 per cent. Experiments were therefore planned to throw some light upon the influence of the moisture content of soil on the effectiveness of carbon disulphid and toluol in killing Azotobacter.

In these experiments the same soil was employed as in the second set. Three conditions of the soil with respect to moisture were compared. In the first the water content was 3 per cent, in the second 10 per cent, and in the third 20 per cent. The soil was made up to different moisture contents before treatment.

After the chemical was allowed to act for three days all samples were cultured for Azotobacter. The bottles were then opened and left open

until the soil became approximately air-dry. This was two days for the first and second sets and six days for the third set. During this time, in order to increase the surface exposed to air and also prevent contamination, the bottles were left lying on their sides under a hood.

After exposing the samples for the chemical to evaporate, another series of cultures were made for Azotobacter. The water content of the soil was then made up to one-half saturation and incubated at room temperature. The water lost by evaporation was restored from time to time. At the end of three months' incubation a third series of cultures for Azotobacter were made and the nitrate content determined.

The actual quantities of nitrogen fixed were not determined, for reasons already given, the character of growth being considered a sufficient index. The results are presented in Table IV.

TABLE IV.—*Effect of carbon disulphid and toluol upon Azotobacter and nitrate accumulation in soil*

WATER CONTENT OF SOIL, 3 PER CENT

Treatment.	Azotobacter film.			NO_3[b]	NH_3
	1[a]	2[a]	3[a]		
				Mgm.	
Control, 0 cc	Typical	Typical	Typical	13.2	Trace.
Do.	do	do	do	12.0	Do.
Carbon disulphid:					
0.10 cc	do	do	do	12.0	Do.
0.10 cc	do	do	do	12.0	Do.
0.25 cc	do	do	do	12.0	Do.
0.25 cc	do	do	do	12.6	Do.
0.50 cc	do	do	do	12.0	Do.
0.50 cc	do	do	do	12.0	Do.
1.00 cc	do	do	do	11.4	Do.
1.00 cc	do	do	do	12.0	Do.
5.00 cc	do	do	do	12.6	Do.
5.00 cc	do	do	do	11.7	Do.
Toluol:					
0.10 cc	do	do	do	12.6	Do.
0.10 cc	do	do	do	12.6	Do.
0.25 cc	do	do	do	12.0	Do.
0.25 cc	do	do	do	11.7	Do.
0.50 cc	do	do	do	10.0	Do.
0.50 cc	do	do	do	11.0	Do.
1.00 cc	do	do	do	12.0	Do.
1.00 cc	do	do	do	12.6	Do.
5.00 cc	do	do	do	12.4	Do.
5.00 cc	do	do	do	12.6	Do.

[a] 1, 2, and 3 have reference to time at which cultures were made. 1 was made after chemical had remained in soil three days, 2 was made after chemical was evaporated, and 3 was cultured after nine weeks' incubation. Nitrates and ammonia were determined after nine weeks' incubation.

[b] Nitrates in milligrams per 100 gm. of soil.

TABLE IV.—*Effect of carbon disulphid and toluol upon Azotobacter and nitrate accumulation in soil*—Continued

WATER CONTENT OF SOIL, 10 PER CENT

Treatment.	Azotobacter film.			NO_3.	NH_3.
	1[a]	2[a]	3[a]		
				Mgm.	
Control, 0 cc	Typical	Typical	Typical	15.0	Trace.
Do.	do.	do.	do.	15.5	Do.
Carbon disulphid:					
0.10 cc	do.	do.	do.	15.0	Do.
0.10 cc	do.	do.	do.	15.0	Do.
0.25 cc	do.	do.	do.	16.5	Do.
0.25 cc	do.	do.	do.	17.2	Do.
0.50 cc	do.	do.	do.	15.5	Do.
0.50 cc	do.	do.	do.	17.2	Do.
1.00 cc	do.	do.	do.	16.0	Do.
1.00 cc	do.	do.	do.	16.0	Do.
5.00 cc	None	None	None	6.4	Abundant.
5.00 cc	do.	do.	do.	15.0	Do.
Toluol:					
0.10 cc	Typical	Typical	Typical	16.0	Trace.
0.10 cc	do.	do.	do.	16.0	Do.
0.25 cc	do.	do.	do.	15.0	Do.
0.25 cc	do.	do.	do.	15.0	Do.
0.50 cc	do.	do.	do.	16.5	Do.[a]
0.50 cc	(?)	(?)	(?)	15.5	Do.
1.00 cc	None	None	None	14.4	Do.
1.00 cc	do.	do.	do.	15.0	Do.
5.00 cc	do.	do.	do.	12.0	Abundant.
5.00 cc	do.	do.	do.	9.0	Do.

WATER CONTENT OF SOIL, 20 PER CENT

Control, 0 cc	Typical	Typical	Typical	12.6	Trace.
Do.	do.	do.	do.	12.6	Do.
Carbon disulphid:					
0.10 cc	do.	do.	do.	12.7	Do.
0.10 cc	do.	do.	do.	12.6	Do.
0.10 cc	do.	do.	do.	13.0	Do.
0.25 cc	do.	do.	do.	13.0	Do.
0.25 cc	do.	do.	do.	13.2	Do.
0.50 cc	do.	do.	do.	12.0	Do.
0.50 cc	do.	do.	do.	11.4	Slight.
1.00 cc	do.	do.	do.	12.0	Do.
1.00 cc	do.	do.	do.	5.5	Abundant.
5.00 cc	None	None	None	6.0	Do.
5.00 cc	do.	do.	do.		
Toluol:					
0.10 cc	Typical	Typical	Typical	12.6	Trace.
0.10 cc	do.	do.	do.	16.0	Do.
0.25 cc	do.	do.	do.	13.0	Do.
0.25 cc	do.	do.	do.	12.2	Do.
0.50 cc	do.	do.	do.	13.0	Do.
0.50 cc	(?)	(?)	do.	13.2	Do.
1.00 cc	None	None	None	13.0	Do.
1.00 cc	do.	do.	do.	13.2	Abundant.
5.00 cc	do.	do.	do.	10.9	Do.
5.00 cc	do.	do.	do.	9.6	

[a] 1, 2, and 3 have reference to time at which cultures were made. 1 was made after chemical had remained in soil three days, 2 was made after chemical was evaporated, and 3 was cultured after nine weeks' incubation. [illegible] were determined after nine weeks' incubation.

In another experiment a local soil which had shown vigorous Azotobacter development and nitrogen fixation was used. The moisture content varied in different samples, being 3 per cent, 12 per cent, 24 per cent, and 36 per cent, respectively. After three days' treatment with carbon disulphid and toluol the samples to be evaporated were treated as in the preceding experiment. The moisture content of those samples containing 24 per cent of water decreased very slowly, it being necessary to leave them for two weeks before they were air-dry. The other evaporated samples were left the same length of time before the moisture was made up to the required amount. No attempt was made to evaporate the samples containing 36 per cent of water. After evaporation the moisture content of all samples, except those containing 36 per cent, was made up to 24 per cent. During incubation the loss of water due to evaporation was restored.

Cultures for Azotobacter were made six weeks after the soil had been treated, and seven weeks later the nitrate content was determined. A number of quantitative nitrogen determinations were lost through accident, but the qualitative results are sufficient for present needs. The results are given in Tables V and VI.

TABLE V.—*Effect of carbon disulphid upon nitrogen fixation and nitrate accumulation*

NOT EVAPORATED

Treatment.	Water content, 3 per cent.				Water content, 12 per cent.			
	Azotobacter film.	Nitrogen fixed.[a]	NO_3[b]	NH_3	Azotobacter film.	Nitrogen fixed.[a]	NO_3[b]	NH_3
		Mgm.	*Mgm.*			*Mgm.*	*Mgm.*	
Control, 0 cc	Typical	9.10	30.8	Trace	Typical	9.05	25.0	Trace.
Do	do	10.40	33.3	do	do	8.75	24.9	Do.
Carbon disulphid:								
0.25 cc					do	8.65	23.4	Do.
0.25 cc					do	9.35	25.8	Do.
0.50 cc	Typical	10.90	33.3	Trace	do	8.75	24.0	Do.
0.50 cc	do	9.55			do	8.70	24.9	Do.
1.00 cc	do	10.20	35.4	Trace	do	9.30	27.2	Do.
1.00 cc	do	9.30	33.3	do	do	9.30	25.8	Do.
5.00 cc	do	9.20	35.0	do	None	1.90	27.2	Do.
5.00 cc	None	4.65	32.1	do	do	2.15	15.0	Abundant.
10.00 cc	do	2.25	23.4	Abundant.				
10.00 cc	do	1.70	18.6	4.3 Mgm.				

EVAPORATED

Control, 0 cc	Typical	10.50	30.0	Trace	Typical	8.65	27.2	Trace.
Do	do	9.40	34.8	do	do	8.75	30.0	Do.
Carbon disulphid:								
0.25 cc					do	8.90	27.2	Do.
0.25 cc					do	9.25	33.3	Do.
0.50 cc	Typical	9.65	35.4	Trace	do	9.90	33.3	Do.
0.50 cc	do	9.80	33.3	do	do	9.45	28.2	Do.
1.00 cc	do	9.95	33.3	do	do	10.25	33.3	Do.
1.00 cc	do	9.45	33.3	do	do	9.75	28.2	Do.
5.00 cc	do	9.15	35.4	do	(?)	6.45	37.5	Do.
5.00 cc	do	10.55	33.3	do	None	2.45	17.4	Abundant.
10.00 cc	None	4.25						
10.00 cc	do	2.30	30.0	Trace				

[a] Nitrogen fixed in milligrams per 50 cc. of culture. [b] Nitrates in milligrams per 100 gm. of soil.

TABLE V.—*Effect of carbon disulphid upon nitrogen fixation and nitrate accumulation*—Continued

NOT EVAPORATED

Treatment.	Water content, 24 per cent.				Water content, 36 per cent.			
	Azotobacter film.	Nitrogen fixed.[a]	NO_3[b]	NH_3.	Azotobacter film.	Nitrogen fixed.	NO_3.	NH_3.
		Mgm.	*Mgm.*			*Mgm.*	*Mgm.*	
Control, 0 cc	Typical	9.30	37.5	Trace	Typical	9.40	42.8	Trace.
Do	do	9.75	32.1	do	do	9.50	37.5	Do.
Carbon disulphid:								
0.25 cc	do	9.55	37.5	do	do	8.85	37.5	Slight.
0.25 cc	do	9.20	33.3	do	do	8.45	40.8	Trace.
0.50 cc	do	9.50	35.4	do	(?)	3.25	40.0	Do.
0.50 cc	do	8.65	31.0	do	Typical	9.30	42.8	Do.
1.00 cc	do	10.30	33.3	do	(?)	5.95	42.8	Do.
1.00 cc	do	9.65	33.3	do	Typical	9.40	36.0	Do.
5.00 cc	None	3.10	20.0	Abundant	None	2.40	23.4	Abundant.
5.00 cc	do	2.45	18.8	5.0 Mgm	do	2.35	20.4	4.8 Mgm.
10.00 cc								
10.00 cc								

EVAPORATED

Control, 0 cc	Typical	9.15	37.5	Trace				
Do	do	9.25	36.0	do				
Carbon disulphid:								
0.25 cc	do	9.10	38.8	do				
0.25 cc	do	9.85	32.1	do				
0.50 cc	do	9.90	35.4	do				
0.50 cc	do	8.00	34.8	do				
1.00 cc	do	9.35	36.4	do				
1.00 cc	do	8.20	34.8	do				
5.00 cc	None	1.85						
5.00 cc	do	2.05	19.6	5.4 Mgm				
10.00 cc								
10.00 cc								

[a] Nitrogen fixed in milligrams per 50 cc. of culture. [b] Nitrates in milligrams per 100 gm. of soil.

TABLE VI.—*Effect of toluol upon nitrogen fixation and nitrate accumulation*

NOT EVAPORATED

Treatment.	Water content, 3 per cent.				Water content, 12 per cent.			
	Azotobacter film.	Nitrogen fixed.[a]	NO_3[b].	NH_3.	Azotobacter film.	Nitrogen fixed.[a]	NO_3[b].	NH_3.
		Mgm.	*Mgm.*			*Mgm.*	*Mgm.*	
Control, 0 cc	Typical	9.10	30.8	Trace	Typical	9.05	25.0	Trace.
Do	do	9.90	33.3	do	do	8.75	24.9	Do.
Toluol:								
0.25 cc					do		35.0	Do.
0.25 cc					do		28.2	Do.
0.50 cc	Typical	10.00	37.5	Trace	do	9.70	35.4	Do.
0.50 cc	do	10.55	34.8	do	do			
1.00 cc	do		37.5	do	do	10.90	33.3	Trace.
1.00 cc	do		39.1	do	(?)	4.75	33.3	Do.
5.00 cc	None		37.5	Slight	None	3.20	23.4	Abundant.
5.00 cc	do		34.8	0.8 mgm	do	2.40	20.4	4.6 mgm.
10.00 cc	do	2.05	21.4	Abundant				
10.00 cc	do		18.8	4.8 mgm				

[a] Nitrogen fixed in milligrams per 50 cc. of culture. [b] Nitrates in milligrams per 100 gm. of soil.

TABLE VI.—*Effect of toluol upon nitrogen fixation and nitrate accumulation*—Contd.

EVAPORATED

Treatment.	Water content, 3 per cent.				Water content, 12 per cent.			
	Azotobacter film.	Nitrogen fixed.[a]	NO_3[b].	NH_3.	Azotobacter film.	Nitrogen fixed.[a]	NO_3[b].	NH_3.
		Mgm.	*Mgm.*			*Mgm.*	*Mgm.*	
Control, 0 cc	Typical	10.50	30.0	Trace	Typical	8.65	27.2	Trace.
Do	do	9.40	34.8	do	do	8.75	30.0	Do.
Toluol:								
0.25 cc					do	9.80	37.5	Do.
0.25 cc					do	8.70	32.1	Do.
0.50 cc	Typical		33.3	Trace	do	9.60	40.0	Do.
0.50 cc	do		32.1	do	do	8.65	38.4	Do.
1.00 cc	do		33.3	do	None	2.05	37.5	Do.
1.00 cc	do		31.0	do	do	2.80	21.4	Abundant.
5.00 cc	do		27.2	do	do	2.25	21.4	Do.
5.00 cc	do		30.0	do	do	3.25	18.0	5.7 mgm.
10.00 cc	do							
10.00 cc	do		28.6	Trace				

NOT EVAPORATED

Treatment.	Water content, 24 per cent.				Water content, 36 per cent.			
	Azotobacter film.	Nitrogen fixed.[a]	NO_3[b].	NH_3.	Azotobacter film.	Nitrogen fixed.[a]	NO_3[b].	NH_3.
		Mgm.	*Mgm.*			*Mgm.*	*Mgm.*	
Control, 0 cc	Typical	9.30	37.5	Trace	Typical	9.40	42.8	Trace.
Do	do	9.75	32.1	do	do	9.50	37.5	Do.
Toluol:								
0.25 cc	do	9.40	37.5	do	None	5.85	45.0	Do.
0.25 cc	do	9.50	34.8	do	do	4.30	45.0	Do.
0.50 cc	do	9.80	37.5	do	do	3.75	46.2	Do.
0.50 cc	do	10.00	34.8	do	do	1.75	47.4	Do.
1.00 cc	None	2.75	38.8	do	do	2.20	30.0	Abundant.
1.00 cc	do	2.60	34.8	do	do	2.00	33.3	3.6 mgm.
5.00 cc	do	2.00	25.0	Abundant	do	1.75	23.4	Abundant.
5.00 cc	do	2.30	22.5	5.7 mgm	do	2.20	22.5	6.6 mgm.
10.00 cc								
10.00 cc								

EVAPORATED

Control, 0 cc	Typical	9.15	37.5	Trace
Do	do	9.25	36.0	do
Toluol:				
0.25 cc	do	9.75	37.5	do
0.25 cc	do	9.35	34.8	do
0.50 cc	do	9.60	37.5	do
0.50 cc	do	9.85	37.5	do
1.00 cc	None	2.80	37.5	Slight
1.00 cc	do	2.50	34.8	0.9 mgm
5.00 cc	do	2.20	20.0	Abundant
5.00 cc	do	2.25	18.8	5.4 mgm
10.00 cc				
10.00 cc				

[a] Nitrogen fixed in milligrams per 50 cc. of culture. [b] Nitrates in milligrams per 100 gm. of soil.

It will be observed that in the experiments the results of which are presented in Table IV the highest quantity of carbon disulphid and toluol used was without effect upon either Azotobacter or nitrate accum-

ulation in the air-dry soil. For the soil containing 10 per cent of water 5 cc. of carbon disulphid and 1 cc. of toluol were sufficient to destroy Azotobacter, while 5 cc. of either were sufficient to check nitrate accumulation; 0.5 cc. of toluol checked Azotobacter in one sample, but was not sufficient to kill. The results for the soil containing 20 per cent of water are in every respect similar to that containing 10 per cent. In all cases there was an accumulation of ammonia only when nitrification was checked.

The only air-dry samples in which carbon disulphid destroyed Azotobacter were those treated with 10 cc. In one unevaporated sample 5 cc. eliminated Azotobacter, although in the duplicate no elimination occurred. Ten cc. checked the nitrate accumulation in unevaporated samples, but were ineffective where the carbon disulphid was evaporated.

When the water content was 12 per cent, 5 cc. of carbon disulphid were sufficient to destroy Azotobacter and to check nitrate accumulation. This also is true of the soil containing 24 per cent of water. Where the water content was 36 per cent, the results are somewhat irregular. Azotobacter were destroyed and nitrate accumulation was checked when treated with 5 cc.; however, 0.5 and 1.0 cc. appeared to affect the Azotobacter in one instance each, but was without effect upon the nitrate accumulation. This irregularity was probably due to reinoculation in the case of those samples in which duplicates did not agree. The results agree in showing an accumulation of ammonia only in those samples in which nitrification was checked.

The results with toluol as given in Table VI show that in the air-dry soil 5 cc. were sufficient to destroy Azotobacter in the nonevaporated samples, while 10 cc. were insufficient in the evaporated samples. Apparently nitrate accumulation was checked to an appreciable extent with 10 cc. in nonevaporated samples, but was not affected when the toluol was evaporated from the soil. One cc. of toluol was sufficient to destroy Azotobacter in evaporated and in one nonevaporated sample when the water content was 12 per cent, while 5 cc. were required to affectively check nitrate accumulation. In one evaporated sample 1 cc. checked the nitrate accumulation.

When the moisture content was raised to 24 per cent, 1 cc. destroyed Azotobacter in all samples, while 5 cc. were still required to check nitrate accumulation to any appreciable extent.

With a moisture content of 36 per cent, 0.25 cc. of toluol destroyed Azotobacter, and 1 cc. was sufficient to check nitrate accumulation.

As in all other experiments ammonia accumulated only when nitrification had been checked.

It appears from these results that both carbon disulphid and toluol will check nitrate accumulation if applied in sufficient quantities. The quantity necessary to bring about this effect varies quite widely with [illegible] of the soil and probably also with different soils.

The quantity necessary to destroy the nitrifying organisms is very much larger than that which is necessary to check their activilty. There are some times slight increases in the accumulation of nitrate is treated as compared to nontreated soils. This increase, however, can not be attributed to a stimulation of the nitrifying organisms.

There is never an accumulation of ammonia unless nitrification has been checked, whicfi means that in no instance does the process of ammonia formation exceed that of nitrification, the latter process being limited by the former. Any increase in nitrate formation, therefore, merely means an increased ammonia formation. In those samples in which nitrification has been checked and there is an accumulation of ammonia the total nitrogen present as nitrate and ammonia is not materially different from that present in other samples.

The effect of carbon disulphid and toluol upon the Azotobacter group of organisms is more definite and pronounced than it is upon the nitrifying organisms. This is due to the fact that if the application is sufficient to have any appreciable effect, it appears to actually destroy the organisms rather than to check their activity.

Again the effect of both carbon disulphid and toluol appears to depend more upon the condition of the soil than upon the quantity applied. The data show that under some conditions an application of many times as much is necessary to destroy Azotobacter as under other conditions. For these reasons no definite statement can be made as to the effect a given application will have. One-tenth cc. of carbon disulphid per 100 gm. of soil has been observed to destroy Azotobacter completely, while in other instances 5 cc. were without effect. Similarly 0.25 cc. of toluol has been observed to kill, while under other conditions 10 cc. were without effect.

The elimination of Azotobacter does not eliminate nitrogen fixation. There are other nitrogen-fixing organisms that are able to withstand the heaviest application of carbon disulphid and toluol we have made. The quantity of nitrogen fixed in the absence of Azotobacter is usually from one-fourth to one-half that when they are present, and the quantity fixed appears to decrease as the application of carbon disulphid and toluol increases.

As soon as the wide difference in the quantity of carbon disulphid and toluol necessary to destroy Azotobacter under different soil conditions was observed, the question of the cause of such differences very naturally arose. As mentioned in the experimental part of this paper, the moisture content offered a working basis.

In commenting upon the writer's earlier work with carbon disulphid and toluol Kelley [1] suggested that, since these substances are only slightly miscible with water, the noneffectiveness of these agents in decreasing the number of bacteria was possibly due to the high-moisture

[1] KELLEY, W. P. AMMONIFICATION AND NITRIFICATION IN HAWAIIAN SOILS. Hawaii Agr. Exp. Sta Bul. 37, 52 p. 1915.

content used in our experiments. The high-moisture content, according to Kelley, would prevent carbon disulphid and toluol from coming in contact with the organisms. The results herein reported appear to show that this is not true.

A study of the literature devoted to the question of partial sterilization by means of volatile antiseptics reveals a mass of conflicting data and numerous contradictions and criticisms. As far as ascertained, the influence of moisture upon such treatment has been considered by only one investigator. Greig-Smith [1] found that the effct of toluol upon soil protozoa varied with moisture content. The available review of this paper gives no data except the statement that 20 per cent of toluol failed to destroy certain protozoa if the moisture content were less than one-tenth saturation, but if the moisture content was above this 1 or 2 per cent of toluol was sufficient to kill them. In the soils used any degree of saturation below one-tenth would approach very closely an air-dry condition. It appears, then, that the experiments reported in this paper agree in general with Greig-Smith's. It is possible that many of the conflicting results heretofore reported as to the effect of volatile antiseptics, or so-called partial sterilization, may be explained solely upon the moisture content of soil when such treatment was made.

SUMMARY

Carbon disulphid and toluol when applied to soils in sufficient quantities will destroy Azotobacter group of organisms and check the accumulation of nitrate nitrogen and possibly will destroy the nitrifying organisms.

The quantities necessary to produce such effects vary quite widely depending among other things upon the quantity of moisture present.

Apparently if the quantity of carbon disulphid or toluol is sufficient to have any effect upon Azotobacter they are usually completely destroyed. On the other hand, there is a great difference in the quantity necessary to destroy nitrifying organisms and that necessary to check their activity.

There are nitrogen-fixing organisms other than Azotobacter present in soils which are not destroyed with 10 cc. of carbon disulphid or toluol even when the moisture content of the soil is high.

Following treatment with carbon disulphid and toluol there is no appreciable accumulation of ammonia unless nitrification has been checked.

There is no evidence in these experiments to show that treatment with antiseptics stimulates the nitrifying organisms, and there is little evidence to indicate a stimulative effect upon the ammonifying or nitrogen-fixing organisms.

[1] GREIG-SMITH, R. THE ACTION OF TOLUENE UPON SOIL PROTOZOA. (Abstract.) In Nature [London], v. 94, no. 2360, p. 581. 1915.

A MULTIPLE-PIPETTE HOLDER FOR THE DISTRIBUTION OF SERUM FOR THE COMPLEMENT-FIXATION TEST

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DISADVANTAGES OF FORMER METHOD

Those engaged in the performance of complement-fixation tests, especially in laboratories where such work is conducted on a large scale, readily can appreciate the necessity for the so-called "short-cuts" consistent, of course, with accuracy, and one's imagination can readily appreciate the tediousness of removing the serums for diagnosis from about 1,000 (daily average) and at times as many as 2,400 specimens. The transferring of the serums from the bottles to the test tubes has been long a matter of concern and required the employment of many assistants, and even then it would consume the greater part, if not all, of the forenoon in the distribution of the large number of samples.

It might be stated here that the desired quantities of serum are placed in test tubes containing 1.5 cc. of physiological salt solution and inactivated for half an hour at 58° C, the other ingredients being added thereto later.

The pipettes employed in the measuring of the necessary amounts of serum were those of the 1-cc. variety, made of glass, and graduated into tenths and hundredths. Such pipettes are filled by suction with the mouth and controlled by the index finger, and although one may become quite expert in the handling of the same, the constant reading of the smaller graduations is not conducive to good vision. Such pipettes are not infrequently of a larger diameter than the opening in the serum bottle, their use in such cases requiring the uptilting of the bottle with consequent agitation of its contents, a feature not at all desirable; and, further, the utilization of this method of serum measurement meant the consuming of several hours and the assistance of numerous operators.

Necessity therefore prompted the creation of a device which has overcome all the objectionable features above enumerated and has made possible the transferring of serums from bottles to test tubes a matter of comparative ease, requiring but a short space of time and demanding but few operators, all of which are especially desirable in this era of time and labor saving.

After deliberation it was concluded that the new device now utilized and illustrated herein would satisfactorily meet the existing conditions

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and was therefore adopted. It is based on the principle of the multiple pipette devised by Buck [1] which is used in this laboratory for the distribution of salt solution, complement, etc.

DETAILS OF NEW DEVICE

The device (fig. 1) consists of a brass tube, 15¼ inches in length and ⅜ inch in diameter (outside), which is tapped at both ends to permit cleaning should occasion arise. The lumen communicates, in its length, with 12 brass collaterals set at equal distances apart and at right angles to the main tube. Each collateral is 2⅜ inches in length with a ¼-inch lumen, and is lined with rubber tubing of sufficient length to project about ½ inch. This free end of rubber tubing is everted over the opening of the metal collateral, thus causing a slight constriction of the lumen of the rubber at that point and serving to fit snugly about the glass pipettes to be fitted therein. On the opposite side of the main tube, at the center and at right angles, is located a single short metal collateral, or mouth piece, to which is attached a piece of rubber tubing for the control of the device and to which a pinch clamp may be applied, although controlling by pressure with the forefinger and thumb has been found to be more convenient.

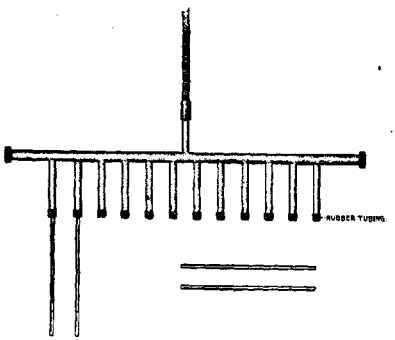

FIG. 1.—General view of device and pipettes.

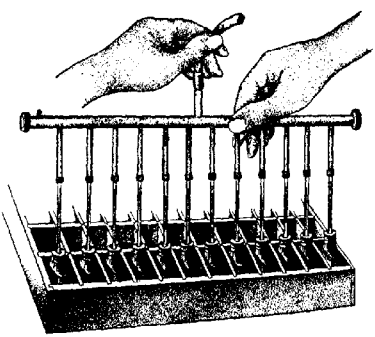

FIG. 2.—Holder connected with serum bottles in standard tray.

The pipettes are made of selected 4-mm. glass tubing and graduated for the proper amount of serum for use in the test. These graduations for convenience are etched at both ends.

The bottles in which the serums are received, although varying slightly in size and shape, have permitted the making of standard trays (fig. 2), so constructed as to hold 144 bottles, 12 bottles wide and deep, and so placed as to register with the pipettes when they are brought into apposition.

Each tray is numbered on the end to indicate its position in the test, while the horizontal rows of bottles are given subnumbers to correspond with their respective test-tube racks. The test tubes are numbered to correspond with the bottles.

The test-tube racks (fig. 3) are constructed to accommodate a double row of 12 tubes each and which, in turn, register with the pipettes. In short, the arrangement of the bottle trays, test-tube racks, and pipettes is standardized.

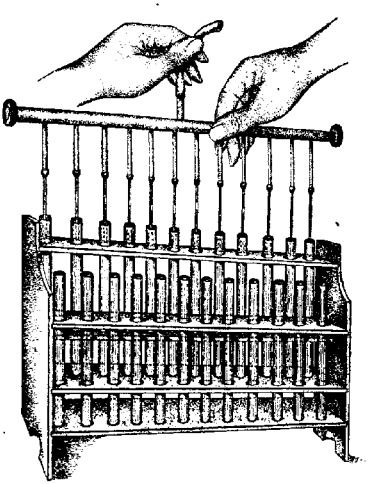

FIG. 3.—Test tubes in rack.

When the stoppers of the serum bottles have been removed and the trays arranged in proper order to correspond with the racks, the operator is handed one of the metal pipette holders containing 12 clean, sterilized pipettes. These are inserted into the 12 bottles of the first row and immersed to just below the surface of the smaller amount when suction is applied to the mouthpiece, and serum from 12 test animals drawn up simultaneously by one operation. The serums are drawn above the etched graduations, then allowed to recede until the graduations are reached. This is done to equalize the amount in each pipette, for it has been found that some bottles contain more than others and when the serums are drawn up the corresponding columns will be higher, but by letting the serum partially escape down to the graduations, the quantities in the pipettes are readily equalized. The serums are then transferred to the 12 test tubes bearing numbers corresponding with those on the bottles.

The holder is then passed to an assistant who removes the used pipettes which are placed in water to prevent drying of the serum, after which fresh pipettes are inserted and equalized by slight pressure on a flat level surface. The use of four metal holders insures always a clean set of pipettes ready for use by the operator.

On completion of the removal of serums, the pipettes are subjected to a thorough washing, drying, and sterilization in hot air for two hours.

Actual tests showed that 1,000 specimens can be removed conveniently from the bottles and placed in test tubes in about one-half hour.

The following advantages are claimed for the holder:

(1) No eyestrain attends the operation of the device.

(2) The pipettes are sufficiently small to enter the bottles without agitation of the contents.

(3) Only one operator and two assistants are required, where previously many were necessary.

(4) Only a short time is needed for one operator to remove specimens of a great number of serum samples.